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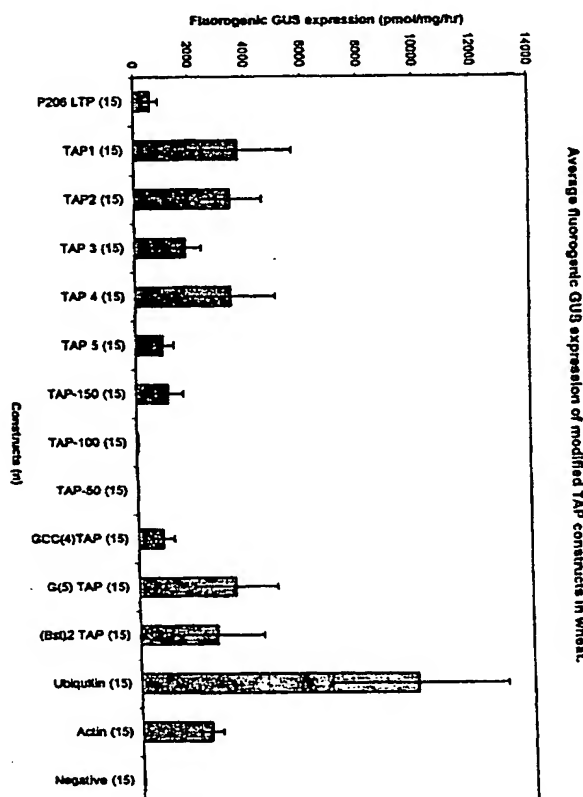
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(54) Title: REGULATORY REGION OF A LIPID TRANSFER PROTEIN (LTPW1) FROM ALEURON TISSUE OF WHEAT



(57) Abstract: This invention is directed to a regulatory re-
gion obtained from a wheat aleurone gene LtpW1. This re-
gulatory region, truncated derivatives, mutations, or deletions
of this regulatory region, can be used to express heterologous
genes of interest within aleurone cells of a plant. Furthermore,
this invention is directed to a truncated LtpW1 regulatory re-
gion that exhibits constitutive activity within both monocot and
dicot plants. This invention is also directed to vectors com-
prising these regulatory regions operatively linked with a he-
terologous gene of interest, as well as plant cell cultures and
transgenic plants comprising these vectors. A method for the
preparation of a plant using the regulatory regions of this in-
vention are also disclosed.

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REGULATORY REGION OF A LIPID TRANSFER PROTEIN (LTPW1) FROM ALEURON TISSUE OF
WHEAT**BACKGROUND OF THE INVENTION**

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The present invention relates to plant gene regulatory regions and their use in the expression of genes of interest. More specifically, the present invention relates to the use of an aleurone regulatory region for organ, and tissue specific expression of a gene of interest within aleurone tissues of plants, and for constitutive expression of a
10 gene of interest within monocot and dicotyledonous plants. This invention also pertains to derivatives of the aleurone regulatory region and their activity in monocot and dicotyledonous plants.

The endosperm of a seed is the site of deposition of storage products such as
15 starch and proteins used by the developing embryo during germination. The endosperm surrounds the embryo of developing and mature cereal seeds. The endosperm comprises a peripheral layer of aleurone cells, which are specialized secretory cells. During germination, the aleurone layer is involved in the transfer of metabolites from the transport system to the endosperm. Furthermore, several antimicrobial compounds
20 required to protect the seed during dormancy, imbibition and germination are synthesized within this tissue. The aleurone cells differentiate from primary endosperm cells 10-21 days after fertilization.

Several aspects of hormonal regulation of gene transcription within aleurone
25 tissue, in germinating barley seeds have been well characterized (Fincher 1989, Annu. Rev. Plant Physiol. Mol. Biol. 40:305-346). For example, genes encoding α -amylase, responsible for the digestion of the starch stored within the starchy endosperm, and β -glucanase, which digests the cell walls, have been isolated and characterized (WO 90/01551 Rogers; US 5,677,474 Rogers, issued October 14, 1997; Karrer et al 1991,
30 Plant Mol. Biol.16:797-805; Slakeski and Fincher 1992). Furthermore, several structural and regulatory genes involved in anthocyanin biosynthesis within the

aleurone have been isolated and characterized (Paz-Ares et al 1987, EMBO J. 5:829-833; Dellaporta et al 1988, pp263-282 18th Stadler Genet. Symp. ed. J.P. Gustafsant and R.Appels). Other genes representing differentially expressed transcripts within aleurone layers have also been reported including CHI26 (Lea et al 1991, J. Biol. Chem. 266:1564-73); pZE40 (Smith et al 1992, Plant Mol. Biol. 20:255-66); pHvGS-1, and pcHth3 (Heck and Ho 1996, Plant Mol. Biol. 30:611-23). Several genes encoding lipid transfer proteins (Ltp) have also been obtained from barley aleurone tissues, including B11E- barley *Ltp1*, and B12A- barley *Ltp2* (Jakobsen et al 1989, Plant Mol. Biol. 12: 285-93). Only one of these genes, B12A, has been expressed ectopically in transgenic plants. In this case the promoter is active only during seed development (Kalla et al 1994 Plant J. 6:849-860)

Lipid transfer proteins are responsible for the transfer of phospholipids between membranes *in vitro*, and likely play a role during membrane biogenesis. This may be especially important in aleurone cells which are known to develop extensive membrane systems. Skriver et al (1992, Plant Mol.Biol. 18: 585-589) disclose the sequence of a genomic Ltp (*Ltp1*), including the promoter region, from barley. Northern analysis demonstrated that this gene was specifically expressed in developing and germinating seeds, as well as in whole seeds and aleurone layers obtained from seeds 30 days post anthesis (dpa). No expression of *Ltp1* mRNA was observed in root, leaf, or shoot tissues, or coleoptiles of germinating seeds. Linnestad et al (1991, Plant Physiol 97: 841-843) also discloses the promoter sequence of the *Ltp1* promoter from barley which was obtained using barley cDNA B12A as a probe. The *Ltp1* promoter, as well as a modified form of this promoter is disclosed in WO 95/23230 (February 23, 1995; Olsen et al). The modified form of the *Ltp1* promoter was not specific to directing expression within aleurone cells, and was active in a range of plant organs and tissues including aleurone cells, scutellar epithelial tissue and vascular tissue during germination or in the plant, including root, leaves and stem.

The promoter of B12A (also termed *Ltp2*) directs expression specifically within the aleurone layer of developing grain as determined using transgenic cereal plants

(Kalla et al 1994, Plant J. 6: 849-860). The sequence of the Ltp2 promoter is disclosed in CA 2,110,772 (filed December 6, 1993, Olsen and Kalla) and US 5,525,716 (Kalla et al). Dieryck et al (1992, Plt., Molec. Biol 19:707-709) disclose the incomplete cDNA sequence of a wheat (*Triticum durum*) Ltp (pTd4.90). Ltp genes comprise a multigenic family and are ubiquitous in plants. Unfortunately as these genes or corresponding proteins have been isolated from various species there is no uniformity in the terminology used to identify the genes. Hence Ltp1 from tobacco, barley and Arabidopsis are not the same. As well, two barley Ltp2 genes are described in the literature; barley Ltp2 as described in Molina and Garcia-Olmedo (Plant J. 4:983-991) is a leaf Ltp, while barley Ltp2 as described in Kalla et al (1994 Plant J. 6:849-860) is aleurone specific.

It is desirable to provide regulatory regions capable of controlling aleurone specific expression that is not detrimental to the developing embryo and seedling. Aleurone-specific regulatory regions may be used for the regulation of the expression of heterologous or native genes within aleurone tissue of cereal seeds in order to modify grain development and germination. For example, placing genes of interest under the control of aleurone-specific regulatory regions may be used to:

- 1) mediate the unloading of metabolites from the transport system into the endosperm, since this metabolite unloading is processed through aleurone cells. By expressing genes of interest involved in this process specifically within the aleurone, the grain yield may be affected. For example, which is not to be considered limiting in any manner, these genes of interest may include Na^+ and K^+ ATPases functioning in active transport, modifiers of membrane pore exclusion parameters such as TMV movement proteins, invertase for sucrose transport etc.;
- 2) affect the quality of the grain, through the production of specific proteins or enzymes, lipids, secondary metabolites etc. and their secretion into the endosperm during endosperm development or endosperm digestion. For

example, which is not to be considered limiting in any manner, such proteins may include starch synthase, ADP glucose pyrophosphorylase, monoclonal antibodies, glutenins, anticoagulants (eg hirudin), anti-pathogenic phenolics etc.. Furthermore, expression of a gene of interest within the aleurone may also be used in order to express proteins for nutritional or medicinal purposes for feeding to animals or humans;

- 3) regulate pre-harvest sprouting by affecting dormancy, for example which is not to be considered limiting, by over-expression of ACC synthase to induce inhibitory levels of ethylene;
- 4) enhance alcohol production- introduction of novel high temperature resistant enzymes for industrial application, including, but not limited to, thermostable amylases, pectinases and invertase;
- 5) modify disease resistance of developing and germinating grains by expressing proteins, for example but not limited to, oxalate oxidase, glucose oxidase, chitinase, or lipid transfer proteins, in combination with a suitable signal peptide for targeting to the extracellular matrix and cell wall localization. This approach can be used to modify the matrix to provide a stronger physical barrier against invading pathogens or to direct specific anti-pathogen agents to the aleurone/pericarp interface.

This invention characterizes a novel wheat aleurone specific regulatory region active during embryo development and germination and which controls expression of heterologous genes of interest within transgenic plants. Furthermore, this invention relates to a constitutive regulatory element that is active within monocot and dicotyledonous plants, and which can be used to drive the expression of a gene of interest in a variety of plants.

SUMMARY OF THE INVENTION

The present invention relates to plant gene regulatory regions and their use in the expression of genes of interest. More specifically, the present invention relates to the use of a constitutive regulatory region and derivatives of this regulatory region for expression of a gene of interest within both monocotyledonous and dicotyledonous plants.

Accordingly, the present invention is directed to a regulatory element comprising, the nucleotide sequence of SEQ ID NO:3, or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:3 under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity. Preferably, the regulatory element exhibits constitutive activity. Furthermore, the present invention includes the regulatory element as just defined wherein the regulatory element is a chimeric regulatory element, comprising an exogenous regulatory element selected from the group consisting of an enhancer element and a silencer element.

This invention also pertains to a vector comprising the regulatory element defined above in operative association with a gene of interest, and to a transformed plant cell culture, a transgenic plant, either monocotyledonous or dicotyledonous plant, or a transgenic seed comprising the vector as just defined.

The present invention also embraces a regulatory element comprising, a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26, or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and that exhibits regulatory element activity.

This invention relates to a transgenic dicotyledonous plant comprising a gene construct, wherein the gene construct comprises:

- 5 i) a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26 , or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and that exhibits regulatory element activity; and
- 10 ii) a gene of interest in operative association with said nucleotide sequence.

Furthermore, this invention includes a transgenic monocotyledonous plant comprising a gene construct comprising:

- 15 i) a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26 , or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and that exhibits regulatory element activity; and
- 20 ii) a gene of interest in operative association with said nucleotide sequence.

The present invention also considers a method of expressing a gene of interest within a plant comprising:

- 25 i) operatively linking a gene of interest for which expression is desired with the regulatory region of claim 1 to produce a gene construct; and
- ii) introducing said gene construct into said plant and allowing for expression of said gene of interest.

30 This method includes a plant that is a monocotyledonous plant, or a dicotyledonous plant.

This summary of the invention does not necessarily describe all necessary
5 features of the invention but that the invention may also reside in a sub-combination
of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

5

FIGURE 1 shows Ltp expression in aleurone tissues of *Hordeum* and *Triticum* species using Northern analysis hybridized with a barley Ltp1 DIG-labelled cDNA. Figure 1(a) shows *Hordeum vulgare* at 20 dpa; Figure 1(b) shows *Triticum aestivum* at 10 dpa; Figure 1 (c) shows *T. aestivum* at 20 dpa; Figure 1 (d) shows *T. tungidum* at 10 dpa.

10

FIGURE 2 shows RNA *in situ* hybridization of ³⁵S-labelled barley Ltp ribo-probe in 73h germinating, and 18 dpa developing wheat grain. Figure 2 (a) and Figure 2 (b) show 73h germinating wheat grain, and Figure 2 (c) and Figure 2 (d) show 18 dpa developing wheat grain. Figure 2 (a) and Figure 2 (c) show hybridization results using anti-sense probe; Figure 2 (b) and Figure 2 (d) show hybridization with sense probe.

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FIGURE 3 shows the DNA sequence of the genomic LtpW1 gene. The coding region is underlined (the intron is not underlined). The ATG start and TGG stop codon are in bold type. The cap site, TATA, CAT boxes are italicized and double-underlined at positions -83, -117 and -222, respectively. SEQ ID NO 1 runs from -687 to -1, SEQ ID NO 2 runs from -473 to -1, and SEQ ID NO 3 runs from -206 to -1.

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FIGURE 4 shows the DNA sequence alignment of LtpW1 and barley Ltp genes. Figure 4 (a) shows alignment of LtpW1 (top row) and barley Ltp1 (bottom row); Figure 4 (b) shows alignment of LtpW1 (top row) and barley Ltp2 (Kalla et al 1994 Plant J. 6:849-860). The ATG of the Ltp genes is overlined.

30

FIGURE 5 shows the LtpW1 regulatory region constructs, in all three constructs the ADH1S6 intron lies between the LtpW1 regulatory region and the coding region of the marker gene, GUS. Figure 5(a) p687LtpW1-GUS; Figure 5(b) p473LtpW1-GUS; Figure 5(c) p206LtpW1-GUS; Figure 5(d) pLC-GUS, the promoterless control used in transient assays.

FIGURE 6 shows two constructs used for comparative studies containing prior art promoters. Figure 6(a) P35s-GUS, Figure 6(b) pACT-GUS.

FIGURE 7 shows transient expression of LtpW1 regulatory region - GUS (p687LtpW1-GUS) fusion in aleurone of cereal grains delivered by microprojectile bombardment. Figure 7(a) shows *T. aestivum* at 15 dpa; Figure 5(b) shows *Zea mays* at 13 dpa, and Figure 7(c) shows *H. vulgare* at 12 dpa.

FIGURE 8 shows GUS expression in aleurone layer (arrowed) of 3 days germinated kernel of *Z. mays*, T1 self progeny, transformed with p473LtpW1-GUS fusion.

FIGURE 9 shows a restriction map of the LtpW1 regulatory region corresponding to the sequence of SEQ ID NO:1.

FIGURE 10 shows a diagrammatic representation of the p206Ltp-oxo plasmid comprising the 206 fragment of the Ltp regulatory region (Ltp prom), adjoining an intron and the oxo coding region.

FIGURE 11 shows the expression of p206Ltp-oxo in leaf tissue of transgenic corn. CK44 is a non-transformed control, and Act-oxo Homo is an actin-OXO T₅ line homozygous for the OXO gene, and is a high expressing positive control. The remaining T₀ transgenic lines comprise p206Ltp-oxo.

5 **FIGURE 12** shows a series of deletion constructs of the TAP (p206Ltp) regulatory region and there activity in corn. Figure 12(A) shows a diagrammatic representation of the 5' deletion series of the TAP regulatory region fused to the reporter GUS. Figure 12(B) shows the activity of the constructs outlined in Figure 12(A) within corn callus as determined using transient expression analysis.

10 **FIGURE 13** shows a series of mutational TAP constructs. Figure 13 (A) shows a diagrammatical representation of a mutational series TAP 1- TAP 5. Figure 13(B) shows a diagrammatical representation of several chimeric TAP constructs, GCC(4) TAP, G(5) TAP and (Bst2) TAP. Figure 13(C) shows the substituted nucleotides within the TAP1 to TAP 5 mutational series of Figure 13(A). Substitutions 1-5 correspond to TAP 1 to TAP 5, respectively. Figure 13(D) shows the nucleic acid sequence of the deletion series for Figure 13(A).

15

FIGURE 14 shows activity of several regulatory regions of the present invention. Figure 14(A) shows the activity of a mutational series TAP 1 to TAP 5, and several deletion constructs, TAP-150, TAP-100, and TAP-50, and several chimeric regulatory regions, GCC(4)TAP, G(5)TAP, (Bst)2TAP in wheat callus. Figure 14(B) shows the activity of three other modified TAP regulatory regions within wheat callus. Figure 14(C) shows the activity of a mutational series TAP 1 to TAP 5, and several deletion constructs, TAP-150, TAP-100, and TAP-50, and several chimeric regulatory regions, GCC(4)TAP, G(5)TAP, (Bst)2TAP in tobacco leaf cells.

20

DESCRIPTION OF PREFERRED EMBODIMENT

5 The present invention relates to plant gene regulatory regions and their use in the expression of genes of interest. More specifically, the present invention relates to the use of a constitutive regulatory region for expression of a gene of interest within plants.

10 The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

15 Described below is a constitutive regulatory element p206LtpW1 (SEQ ID NO:3), that is active in both monocotyledonous and dicotyledonous plants. The constitutive regulatory element was obtained from a wheat genomic Ltp sequence termed LtpW1 (SEQ ID NO:4). The coding region of the LtpW1 gene sequence exhibits about an 85 % homology with barley Ltp1, and includes a 26 amino acid transit peptide. The regulatory region of a wheat lipid transfer protein (Ltp) gene, LtpW1, has been isolated and characterized. This regulatory region comprises a novel
20 oligonucleotide sequence (SEQ ID NO:1), which is active in aleurone of wheat, maize and barley. The full length regulatory region is not active in leaf, root, or coleoptile tissues. However, a truncated form of LtpW1 regulatory region, termed 206Ltp, 206LtpW1 or TAP (Truncated Aleurone Promoter; SEQ ID NO 3), is active in a range of tissues and plant organs, and is active in both monocot and dicot plants.

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The regulatory region of LtpW1 compared to the barley Ltp1 regulatory region has 43 % sequence similarity with the majority of sequence similarity (82 %) occurring within 140 nucleotides upstream of the transcriptional start site (see Figure 4(a)). A minor sequence similarity was noted between LtpW1 and a barley amylase protease
30 inhibitor, however, no sequence similarity of any significance was observed between LtpW1 and Ltp2, or other known Ltp regulatory region sequences.

The full length LtpW1 regulatory region (687 nucleotides; p687LtpW1; SEQ ID NO:1), or a truncated LtpW1 regulatory region, p473LtpW1 (SEQ ID NO:2; comprising a 473 nucleotide fragment of the full length regulatory region), can be used to drive the expression of a gene of interest within the aleurone layer of a developing and germinating seed of a monocotyledonous plant, for example, but not limited to, wheat, rice, barley and maize.

LtpW1 exhibits 8.8% of 35S activity and 12.2 % activity of the strong rice action monocot constitutive regulatory region (Table 2, Example 3). Comparison of histological evidence of expression of Ltp2 (Kalla et al 1994, Plant J. 6:849-860)), with Figure 7 of the present invention (histological evidence of LtpW1 activity) indicates that LtpW1 is more than two times stronger than Ltp2.

Experiments with deletions of the LtpW1 regulatory region indicate that the 473 nucleotide fragment (SEQ ID NO: 2; p473LtpW1) of the full length regulatory region is more active in aleurone tissue than the 687 base pair regulatory region, (SEQ ID NO: 1), p687LtpW1 (Table 3). However, neither the full length regulatory region, nor the 473 bp truncated regulatory region were active in leaf tissue (see Table 4). A truncated regulatory region comprising a 206 bp nucleotide fragment (SEQ ID NO:3, p206LtpW1, also referred to as TAP) of the full length regulatory region was active in aleurone, leaf, and scutellum tissue, functioning as a minimal regulatory region element. This 206 bp region therefore represents a novel, constitutive, regulatory region useful for expressing a gene of interest within plants.

The 206bp region was also found to direct the expression of a gene of interest within leaf tissue of a range of dicotyledonous plants, including *Soya*, *Brassica* and *Nicotiana* (Table 6, Example 6). Chimeric constructs comprising the strong monocot promoter, the rice actin promoter (McElroy D., Zhang, W., Cao, J., and Wu, R. 1990. Plant Cell 2:163-171) were also introduced into these plants, and no activity was observed. These results indicate that the 206bp region also represents a novel constitutive regulatory region active in a range of plants, including both dicotyledonous

and monocotyledonous plants. Deletion and mutational analysis, and other modifications of the TAP regulatory element, including the preparation of chimeric regulatory elements, indicate that the level of TAP activity can be increased in both monocot and dicot plants (example 7).

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Figure 3 shows the sequence of the LtpW1 gene comprising the regulatory element region as identified in SEQ ID NO:1. The numbering of the regulatory region in Figure 3 is from base pairs -687 to -1, while the coding region of the gene is from base pairs +1 to 753. Therefore, p687LtpW1 comprises the sequence of SEQ ID
10 NO:1 (nucleotides 1-687), which are equivalent to the sequence of base pairs -687 to -1 of Figure 3. p473LtpW1 comprises the sequence of SEQ ID NO:2, nucleotides 214-687 of SEQ ID NO:1, or bps -473 to -1 of Figure 3. p206LtpW1 comprises the sequence of SEQ ID NO:3, nucleotides 481-687 of SEQ ID NO:1, or bps -206 to -1 of Figure 3.

15

By "regulatory element" or "regulatory region", it is meant a portion of nucleic acid typically, but not always, upstream of a gene, and may be comprised of either DNA or RNA, or both DNA and RNA. The regulatory elements of the present invention includes those which are capable of mediating organ specificity, or
20 controlling developmental or temporal gene activation. Furthermore, "regulatory element" includes promoter elements, core promoter elements, elements that are inducible in response to an external stimulus, elements that are activated constitutively, or elements that decrease or increase promoter activity such as negative regulatory elements or transcriptional enhancers, respectively. It is also to be understood that
25 enhancer elements may be repeated thereby further increasing the enhancing effect of an enhancer element on a regulatory region. In the context of this disclosure, the term "regulatory element" also refers to a sequence of DNA, usually, but not always, upstream (5') to the coding sequence of a structural gene, which includes sequences which control the expression of the coding region by providing the recognition for
30 RNA polymerase and/or other factors required for transcription to start at a particular site. An example of a regulatory element that provides for the recognition for RNA

polymerase or other transcriptional factors to ensure initiation at a particular site is a promoter element. A promoter element comprises a core promoter element, responsible for the initiation of transcription, as well as other regulatory elements (as listed above) that modify gene expression. It is to be understood that nucleotide sequences, located within introns, or 3' of the coding region sequence may also contribute to the regulation of expression of a coding region of interest. A regulatory element may also include those elements located downstream (3') to the site of transcription initiation, or within transcribed regions, or both. In the context of the present invention a post-transcriptional regulatory element may include elements that are active following transcription initiation, for example translational and transcriptional enhancers, translational and transcriptional repressors, and mRNA stability determinants.

The regulatory elements, or fragments thereof, of the present invention may be operatively associated with heterologous or exogenous regulatory elements or promoters in order to modulate or mediate the activity of the heterologous regulatory element. Such modulation includes enhancing or repressing transcriptional activity of the heterologous regulatory element, modulating post-transcriptional events, or both enhancing or repressing transcriptional activity of the heterologous regulatory element and modulating post-transcriptional events. For example, one or more regulatory elements, or fragments thereof, of the present invention may be operatively associated with enhancer or silencer elements, to mediate the activity of such regulatory elements within a variety of plants. Furthermore, an intron, for example, but not limited to, the IVS6 intron from maize (Callis, J., 1987, *Genes Dev.* 1: 1183-1200), or the maize actin intron (McElroy, D. et al., 1991. *Mol. Gen. Genet.* 231: 150-160), may be included for optimizing expression within monocotyledonous or dicotyledonous plants.

An "analogue" of the regulatory elements described herein include any substitution, deletion, or additions to the sequence of a regulatory element provided that the analogue maintains at least one regulatory property associated with the activity of the regulatory element. Such regulatory properties include directing expression of

a gene in operative association with the regulatory element in one or more tissues, or directing organ-specific expression, imparting tissue specificity, or a combination thereof, or temporal activity, or developmental activity, or a combination thereof, or other regulatory attributes including, constitutive activity, negative regulatory
5 elements, enhancer sequences, or sequences that affect stability of the transcription or translation complexes or stability of the transcript.

The DNA sequences of the present invention thus include the DNA sequences of SEQ ID NO: 1 to 11, and 22 to 26, the regulatory regions and fragments thereof,
10 as well as analogues of, or nucleic acid sequences comprising about 80% similarity with the nucleic acids as defined in SEQ ID NO's: 1 to 11 or 22 to 26. Analogues (as defined above), include those DNA sequences which hybridize under stringent hybridization conditions (see Maniatis *et al.*, in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982, p. 387-389) to any one of the DNA
15 sequence of SEQ ID NO: 1 to 11 and 22 to 26, provided that said sequences maintain at least one regulatory property of the activity of the regulatory element as defined herein.

An example of one such stringent hybridization conditions may be hybridization
20 in 4XSSC at 65°C, followed by washing in 0.1XSSC at 65°C for an hour. Alternatively an exemplary stringent hybridization condition could be in 50% formamide, 5XSSC at 42°C and washing in from about 0.5XSSC to about 0.2XSSC at 65°C. Analogues include those DNA sequences which hybridize to any one of the sequences of SEQ ID NO: 1 to 11 and 22 to 26 under these hybridization conditions.

25

A constitutive regulatory element directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S transcript. (Odell *et al.*, 1985, *Nature*, 313: 810-812), the rice actin 1 (Zhang *et al.*, 1991, *Plant Cell*, 3: 1155-1165) and triosephosphate isomerase 1 (Xu *et al.*, 1994, *Plant Physiol.* 106: 459-467) genes, the maize ubiquitin 1 gene (Cornejo *et al.*, 1993,
30

Plant Mol. Biol. 29: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995, *Plant Mol. Biol.* 29: 637-646), T1275 (U.S. 5,824,872), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995 *Plant Mol. Biol.* 29: 995-1004). The regulatory element, p206LtpW1, as described herein, is another example
5 of a constitutive regulatory element.

The present invention also includes a chimeric regulatory region comprising the p206Ltp (also referred to as TAP) regulatory region as defined by SEQ ID NO:3, or a nucleic acid that hybridizes to SEQ ID NO: 3, as defined above, a fragment of
10 p206Ltp (TAP) regulatory region, for example but not limited to, fragments as defined by SEQ ID NO's: 5 to 11, or a substituted, modified, or mutated regulatory region, for example but not limited to the regulatory regions defined in SEQ ID NO's 22-26, and one or mediators of this regulatory activity. By "mediate", it is meant the activity associated with an exogenous regulatory element that further regulates the activity of
15 p206Ltp. For example, which is not to be considered limiting in any manner, a mediator may either up regulate, down regulate the activity of TAP, and comprise one or more enhancer or silencer elements, respectively. Examples of enhancer elements are known in the art, and include, but are not limited to, enhancers active in dicotyledonous and monocotyledonous plants, the 35S enhancer, actin enhancer, and
20 the enhancer from T1275 (*Bst*I element; U.S.5,824,872, which is incorporated herein by reference). Furthermore, chimeric regulatory elements comprising p206Ltp or an analogue or fragment thereof may also include, but are no limited to, the addition of one or more GCC boxes, one or more G boxes or other sequences that may otherwise regulate the regulatory region activity of the regulatory region (see for example Figure
25 13(B)). Regulatory region activity associated with such chimeric regulatory regions have been demonstrated in monocots (Figure 14(A)) and dicots (Figure 14 (C)). However, it is to be understood that other chimeric TAP regulatory regions may also be prepared that exhibit desirable regulatory region properties including increasing, or modulating regulatory activity.

30

Nucleic acid constructs comprising a chimeric regulatory region associated with 206LtpW1 were therefore examined to determine if exogenous regulatory regions, mediators, may further regulate the activity of TAP. Transient expression assays indicate that a dicot enhancer element, for example but not limited to an enhancer
5 obtained from tobacco, is active in increasing the activity of 206LtpW1 in plants (e.g. Table 6, Example 6). Similarly, one or more GCC boxes or one or more G boxes are also able to enhance TAP activity (see Figures 14(A) and 14(C)) in both monocot and dicot plants. Therefore, the present invention is also directed to gene constructs comprising a chimeric 206LtpW1 regulatory region, comprising p206 or a fragment
10 or analogue thereof in association with at least one other exogenous regulatory region. The present invention also pertains to the use of a chimeric regulatory region as just described in operative association with a gene of interest.

Any exogenous gene, or gene of interest, can be used and manipulated
15 according to the present invention to result in the expression of the exogenous gene.

By "gene of interest" it is meant any gene that is to be expressed within a host organism. Such a gene of interest may include, but is not limited to, a gene that encodes a protein directed at improving an agronomic trait of the plant, for example
20 but not limited to improving plant defence against pathogens, or resistance to herbicides. A gene of interest may also include, but is not limited to, a gene that encodes a pharmaceutically active protein, for example growth factors, growth regulators, antibodies, antigens, their derivatives useful for immunization or vaccination and the like. Such proteins include, but are not limited to, interleukins,
25 insulin, G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof, interferons, for example, interferon- α , interferon- β , interferon- τ , blood clotting factors, for example, Factor VIII, Factor IX, or tPA or combinations thereof. A gene of interest may also encode an industrial enzyme, protein supplement, nutraceutical, or a value-added product for feed, food, or both feed and food use. Examples of such proteins include,
30 but are not limited to proteases, oxidases, phytases, chitinases, invertases, lipases, cellulases, xylanases, enzymes involved in oil biosynthesis etc.

The chimeric gene construct of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene of the present construct can therefore be used to construct chimeric genes for expression in plants.

The chimeric gene construct of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the regulatory element selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide for resistance to an antibiotic such

as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β -glucuronidase), or luminescence, such as luciferase are useful.

5 The data presented herein indicate that nucleotides 1-214 and 215-481 of SEQ ID NO: 1 are responsible for imparting tissue specificity within this regulatory region, since once the nucleotides 1-481 are removed from the full length regulatory region, tissue specificity is lost (Table 4). It is contemplated that either of these regions may be combined with any suitable regulatory region of interest, for example, which is not
10 to be considered limiting, a minimal, constitutive, or viral promoter etc. in order to obtain aleurone-specific expression of a gene linked thereto. Both of these regions were found to comprise very low sequence similarity with other sequences present within gene sequence databases such as GenBank.

15 Furthermore, the data presented in Table 3 indicates that the region comprising nucleotides 1-481 of SEQ ID NO: 1 is responsible for regulating the strength of regulatory region activity, and includes both silencer- and enhancer-type activities. For example, the fragment comprising nucleotides 215-481 of SEQ ID NO:1 may be used as an enhancer like element as constructs comprising this region (e.g. p473LtpW1)
20 resulted in increased expression when compared with either the full length regulatory region (p687LtpW1) or the truncated regulatory region p206LtpW1 (see Table 3). Similarly, nucleotides 482-687 (of SEQ ID NO:1) also exhibit enhancer-type activity, since constructs comprising this region (p206LtpW1) exhibited higher levels of expression than the full length regulatory region. Therefore, it is contemplated that
25 nucleotides 214-481, or 482-687 of SEQ ID NO:1 may be combined with any suitable regulatory region of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc., in order to obtain both aleurone-specific expression of a gene linked thereto, as well as increased gene expression.

30 The present invention also pertains to the p687LtpW1 regulatory region comprising additional enhancer or silencer elements, or further comprising

modifications to the sequence of these regulatory regions, for example mutations, additions or substitutions as described in , but not limited to those of Figure 13(A), or (B).

5 Similarly, the fragment comprising nucleotides 1-214 comprises silencer-type elements as constructs comprising this region (e.g. p687LtpW1) result in lower levels of expression compared with the levels of expression obtained with either of the truncated regulatory region constructs, p206LtpW1, or p473LtpW1 (see Table 3). It is contemplated that nucleotides 1-214 may be combined with any suitable regulatory
10 region of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc., in order to obtain both aleurone-specific expression of a gene linked thereto, along with reduced gene expression.

 The truncated regulatory region, p473LtpW1, was used to transform maize,
15 where it was noted that this regulatory region was active only in aleurone of developing and germinating cereal grain.

 In transient assays, activity of the 206 bp *HinII*/*BclII* truncated regulatory element (SEQ ID NO3; Figure 3) in non-aleurone tissues was relatively low (7-11%)
20 compared to other constitutive promoters (see Table 4, Example 3). However, the level of stable expression in transformed plants (Fig10) was high, equalling the activity observed for the expression of a gene of interest under the control of a strong monocot regulatory element, the rice actin promoter (McElroy D., Zhang, W., Cao, J., and Wu, R. 1990. *Plant Cell* 2:163-171). Chimeric TAP regulatory regions were also
25 found to be active in directing the expression of a gene of interest in monocot and dicot tissues (see Figures 14 (A) and (C)) . Therefore, it is contemplated that the p206 (TAP) region may also be combined with any suitable regulatory region of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc. in order to obtain aleurone-specific expression of a gene linked thereto.

30

Deletion analysis of the TAP regulatory region (constructs outlined in Figure 12(A)) indicate that from -206 to about -110 (nucleotides 1- 98 of SEQ ID NO:3) of the TAP regulatory region did not have any appreciable effect of the activity of the p206Ltp regulatory region in monocots, for example corn. However, the region
5 between -100 and -110 (nucleotides 90 to 108 of SEQ ID NO:3) was observed to be required for high expression levels.

The -150 TAP (regulatory region defined in SEQ ID NO: 5), -100 TAP (regulatory region defined in SEQ ID NO:10) and -50 TAP (regulatory region defined
10 in SEQ ID NO:11) regulatory region in monocots is shown in Figure 14(A), and in dicots is shown in Figure 14(C). In monocots, high levels of regulatory region activity are observed with the -150 TAP construct (Figure 14(B)), while the removal of the -150 region removed any activity, as indicated with the activity associated with the -100 TAP construct compared with the -150 TAP construct (Figure 14(A)), or the -120 to
15 -80 region (regulatory region defined in SEQ ID NO:12; Figure 14(B)). These results suggest that one or more core regulatory elements reside within this region (i.e. the -150 to -100- region; nucleotides 58 to 108 of SEQ ID NO:3).

Nucleotides 89-96 of SEQ ID NO:3 are AT rich. To determine if this region
20 is involved with the activity of the TAP regulatory element, a construct was prepared comprising substitutions in this region (-130 to -120/-113 to -116 mutation construct, having a regulatory region defined in SEQ ID NO:13). This construct resulted in a decrease in activity in monocots (Figure 14(B)).

25 Analysis of the deletion constructs in dicots indicates that significant activity is associated with the -150 TAP, -100 TAP and the -50 TAP constructs (regulatory regions defined in SEQ ID NO's: 5, 10 and 11, respectively). Since there is no loss of activity with the removal of the -150 to -100, or -50 region, as was observed in monocots, this demonstrates that the sequence from -50 to -1 (nucleotides 158 to 206
30 of SEQ ID NO:3), exhibits regulatory region activity in dicots.

Several alterations and substitutions of the TAP regulatory region were also examined for regulatory region activity. Example of constructs TAP1 to TAP 5, which are not to be considered limiting in any manner, are shown in Figure 13 (A) (regulatory regions defined in SEQ ID NO's: 22-26). The TAP 1 to TAP 4 constructs
5 all exhibit a large increase in activity in monocots when compared with the activity associated with the p206Ltp (TAP) construct. These data demonstrate that the regulatory region activity may be enhanced through the mutation, alteration, or substitution of nucleotides within the TAP sequence. It is also to be understood that the modifications described above to the TAP regulatory region, as well as chimeric
10 regulatory region constructs, may also be included within the full length Ltp regulatory region, including the p687LtpW1 or p473LtpW1 regulatory regions, or fragments thereof.

Analysis of the mutation constructs disclosed in Figures 13 (A) and (B) were
15 also examined in dicots as shown in Figure 14(C). The TAP 1 to TAP 5 constructs all exhibit a similar level, or an increase in the level of regulatory region activity when compared with the activity associated with the p206Ltp (TAP) construct. Chimeric regulatory elements comprising (Bst)2 TAP, GCC (4) TAP or G(5) TAP exhibit an increase in regulatory region activity (Figure 14(C)).

20

Collectively these results demonstrate that substitutions or additions to the Ltp regulatory region, preferably the p206Ltp region, are functional in both monocot and dicot plants, and result in increased activity in both monocot and dicot plants.

25 The gene constructs of the present invention can also include other optional regulatory motifs such as enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include, for example, the enhancer region of the 35S regulatory region, as well as other enhancers obtained from other regulatory regions, and/or the ATG initiation
30 codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The

translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, embryo or shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geierman and Corey, *Plant Molecular Biology*, 2d Ed. (1988); and Miki and Iyer, Fundamentals of Gene Transfer in Plants. In *Plant Metabolism*, 2d Ed. DT. Dennis, DH Turpin, DD Lefebvre, DB Layzell (eds), Addison Wesley, Langmans Ltd. London, pp. 561-579 (1997). The present invention further includes a suitable vector comprising the chimeric gene construct.

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

5 **Example 1: Localization of Ltp1 Expression**

In order to isolate genes which are functional in aleurone of developing and germinating wheat grain, a barley cDNA probe of an aleurone specific lipid transfer protein gene (Ltp1,) was used to indicate activity of similar genes in wheat aleurones
10 during seed development. Northern blot analyses using a DIG -labelled barley cDNA probe showed that Ltp transcripts were present in aleurone tissue 20 dpa (Figures 1 (a) and 1(c)). No activity was detected in early wheat grain development, 10 dpa (Figure 1(b) but could be detected in *T. turgidum* (Fig. 1(d)).

15 *In situ* hybridization (based on a modification of the procedure outlined in Cox and Goldberg, 1998, Analysis of Plant Gene Expression, In Plant Molecular Biology. A Practical Approach, pp. 1-34) performed on cross sections of developing and germinating grain showed that Ltp expression was limited to aleurone cells. A ³⁵S Ltp antisense ribo-probe hybridized strongly to aleurone cells (Figure.2 (a)), whereas no
20 differential hybridization was observed with the sense RNA probe (Figure 2 (b)). Ltp expression was observed throughout grain development after 18 dpa and during germination up to 73 h post-imbibition at which time the endosperm was depleted. No hybridization was observed in developing endosperm, embryo or pericarp tissues (data not shown).

25

Example 2: Genomic DNA of LtpW1

Genomic DNA was isolated from young leaf tissue of hexaploid wheat, (*T. aestivum*) and digested with XbaI. When this DNA was analysed by Southern blot
30 using standard procedures and a DIG-labelled barley Ltp cDNA probe, three loci for the Ltp1 gene (at 1.5, 6.0, and 7.0 kb) were detected. The copy corresponding to the

1.5 kb XbaI band was cloned by screening a λ long C phage library of size-restricted XbaI fragments with a barley Ltp1 cDNA probe.

LtpW1 refers to the Ltp gene contained within the 1.5 kb XbaI digested *T. aestivum* genomic clone, the sequence of which is shown in SEQ ID NO: 4 (also see Figure 3).

The coding sequence of LtpW1 shares 85% DNA identity with the barley Ltp1 (Figure 4 (a)), includes a 26 amino acid transit peptide for cell wall localization of the protein, and has one predicted 88bp intron which is 44 bp shorter than the equivalent barley intron. The nucleotide sequences LtpW1 and barley Ltp 1 promoter (Linnestad 1991) are well conserved for approximately 140 bp upstream of the ATG start codon whereupon they diverge considerably (Figure 4 (a)). The conserved promoter region includes the putative cap and TATA sites but not the proposed CAT site or other regulatory elements (see Figures 3, and 4 (a)).

The nucleotide sequence of the LtpW1 regulatory region exhibits little or no identity with the barley Ltp2 promoter (Figure 4 (b)). The LtpW1 regulatory region was shown to be active in aleurone of developing and germinating cereal grain which is uniquely different from the barley Ltp2 promoter which is only active during grain development but not during germination (Kalla et al 1994).

Example 3: Expression of GUS under the control of LtpW1 aleurone regulatory regions.

p687LtpW1-GUS

A 687 bp XbaI/BcII regulatory region fragment (SEQ ID NO: 1; Figure3) was subcloned from pLtpW1 and fused to a GUS promoterless reporter cassette (pLC-GUS). pLC-GUS was obtained by removing the 35S promoter as a SacI fragment from pZO1016 (designated p35S-GUS herein), which was a gift from R. Sinibaldi,

Sandoz, Ca. A 687 bp XbaI/BclI fragment was isolated from pTALP1 (containing the 1.5 kb XbaI-digested *T. aestivum* genomic clone) and the sticky ends were filled-in with Klenow fragment of DNA polymerase. This fragment was blunt-end ligated into the SmaI site of pLCGUS (see Figure 5(d)), and the orientation of the insert was
5 checked by digesting with BamHI. The activity of this regulatory region was compared with that of the promoterless construct (pLC-GUS) as well as to constructs comprising constitutive CaMV35S and rice action promoters (see Figures 6 (a) and (b), respectively). These constructs were used for comparison of promoter activities. The 35S promoter is described in: Odel, J.T., Nagy, F. and Chua N-H (1985) Nature
10 313:810-812. The rice actin promoter is described in: McElroy D., Zhang, W. Cao, J. and Wu, R. (1990) Plant Cell 2:163-171.

These constructs were introduced into the aleurone of cereal grains by microparticle bombardment using standard methods. LUC and GUS constructs were
15 co-bombarded in equimolar amounts and GUS is expressed relative to LUC to minimize variability between reps (shots). LUC activity serves as an internal control for the shot to shot variability.

Tissues, 48 h post-bombardment, were incubated in reaction buffer containing
20 50mM NaH₂PO₄ (pH 7.0), 10 mM EDTA and 1 mM 5-bromo-4-chloro-3-indolyl-B-glucoronide (X-Gluc), 0.5mMK₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆] at 37°C for 4-20 h. A blue precipitate in the bombarded cells indicates activity of B-glucoronidase. The regulatory region gave high expression of GUS in histological transient assays with wheat aleurones (Figure 7(a)). Activity was also demonstrated in maize and barley
25 aleurones (Figure 7(b) and (c)) The 687 bp regulatory region fragment showed no activity in leaf, root or coleoptile tissues of wheat (data not shown).

In quantitative expression assays in wheat aleurone the 687 bp regulatory region had 3.4% of the activity of the constitutive 35S promoter (Table 1). This
30 underestimates the relative aleurone-directed activity of the LtpW1 regulatory region because of additional endosperm-derived activity of the constitutive 35S promoter.

Table 1: Activity of p687LtpW1 in 12 dpa wheat aleurone

Construct	Luciferase (mv/sec/mg protein)	GUS (pmol MU/min/mg protein)	GUS/LUC	%35S Activity
Au	2001	0	0	-
pLC-GUS ² /p35S-LUC	2100	0	0	-
p35S-GUS/p35S-LUC	3400	30072	8.84	-
p687LtpW1-GUS/p35S-LUC	4200	1247	0.3	3.4

1: mean of three sets of bombardments

2: promoterless construct

P473LtpW1-GUS

15

A truncated version of the LtpW1 regulatory region (see SEQ ID NO:2; nucleotides -473 to -1 of Figure 3; or nucleotides 214-687 of SEQ ID NO:1) was prepared by digesting pTALTP1 with HincII and BclI, and the resulting 0.47kb fragment (after treatment with Klenow) was ligated into the SmaI site of pLC-GUS.

20 Orientation of the insert was checked by digesting the resulting recombinant plasmid with BamHI. The construct (p473LtpW1-GUS) thus obtained showed 8.8% and 12.2% activity of the constitutive 35S and rice action promoters, respectively (pAct-GUS was a gift from Ray Wu at Cornell). See Table 2 for results.

Table 2: Activity of p473LtpW1 in 12 dpa wheat aleurone

	Construct	Luciferase	GUS	GUS/LUC	%35S
		(inv/sec/mg protein)	(pmol MU/min/mg protein)		Action Activity
5	Au	2001	0	0	-
	pLC-GUS ² /p35S-LUC	1300	0	0	-
	p35S-GUS/p35S-LUC	3500	8077	2.31	-
	pAct-GUS/p35S-LUC	3300	5524	1.67	
	p473LtpW1-GUS/p35S-	3200	651	0.2	8.8 12.2
10	LUC				

1: mean of three sets of bombardments

2: promoterless construct

15

When compared within a single experiment, the 473 bp fragment was 170% as active as the 687 bp fragment (Table 3).

Table 3: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in 7 dpa wheat aleurone

	Construct	Luciferase (mv/sec/ mg/protein)	GUS (pmol MU/min/mg protein)	GUS/LUC	%35S Activity
5	pLC-GUS ² /p35S-LUC	97001	120	0.01	0.1
	p35S-GUS/p35S-LUC	2300	18305	7.96	-
	p206LtpW1-GUS/p35S-LUC	7900	1781	0.23	2.9
	p473LtpW1-GUS/p35S-LUC	6800	2399	0.35	4.4
	p687LtpW1-GUS/p35S-LUC	5100	1090	0.21	2.6

10

1: mean of three sets of bombardments

2: promoterless construct

P206LtpW1-GUS

15

To generate p206LtpW1-GUS, pTALTP1 was digested with Bcl1, then with HindIII, and the 0.2 kb fragment was isolated from a gel and purified. The sticky ends were filled in with Klenow and the resulting fragment was ligated into the Sma1 site of pLC-GUS. Neither the 687 bp or 473 bp regulatory regions was active in leaf tissue but the 206 bp HinII/BclI truncated regulatory region (SEQ ID NO:3; nucleotides -206 to -1 of Figure 3; nucleotides 481-687 of SEQ IDNO:1) had 7.5% the activity of the 35S promoter in leaf (Table 4).

20

Table 4: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in wheat leaf tissue

5	Construct	Luciferase	GUS	GUS/LUC	%35S
		(v/sec/mg/ protein)	(pmol MU/min/mg protein)		Activity
	pLC-GUS ² /p35S-LUC	2001	1.6	0.007	0.7
	p35S-GUS/p35S-LUC	200	204.3	1.02	-
	p206LtpW1-GUS/p35S-LUC	200	15.3	0.077	7.5
	p473LtpW1-GUS/p35S-LUC	700	1.3	0.002	0.2
10	p687LtpW1-GUS/p35S-LUC	700	1.7	0.002	0.2

1: mean of three sets of bombardments

2: promoterless construct

15

Similarly, in wheat scutellum tissue, only the 206 bp regulatory region fragment was active (Table 5) with activities of 11.4 % of 35S and 8.5 % of rice actin promoters.

**Table 5: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in 20 dpa
wheat scutellum tissue**

5	Construct	Luciferase	GUS	GUS/LUC	%35S
		(v/sec/mg/ protein)	(pmol MU/min/mg protein)		Action Activity
	pLC-GUS ² /p35S-LUC	13001	37	0.028	0.23, 0.17
	p35S-GUS/p35S-LUC	400	4873	12.182	-
	pAct-GUS/p35S-LUC	400	6530	16.325	-
	p206LtpW1-GUS/p35S-LUC	100	139	1.39	11.41, 8.51
10	p473LtpW1-GUS/p35S-LUC	200	2	0.01	0.08, 0.06
	p687LtpW1-GUS/p35S-LUC	200	6	0.03	0.24, 0.18

1: mean of three sets of bombardments

2: promoterless construct

15

Thus the nucleotide sequence between 206 bp and 473 bp determines the tissue and stage dependent regulation of the LtpW1 regulatory region.

20 Collectively, these data indicate that:

i) nucleotides 1-214 and 215-481 of SEQ ID NO:1 are responsible for imparting tissue specificity within this regulatory region. Removal of either of these regions from the full length regulatory region results in greatly reduced tissue specificity (Table 25 4).

ii) the region comprising nucleotides 1-481 of SEQ ID NO: 1 is responsible for regulating the strength of regulatory region activity, and includes both silencer- and enhancer-type activities:

iii) the fragment comprising nucleotides 214-481 of SEQ ID NO:1 exhibits enhancer-like activity as constructs comprising this region (e.g. p473LtpW1) resulted in increased expression when compared with either the full length regulatory region (p687LtpW1), or the truncated regulatory region p206LtpW1 (see Table 3). Similarly, nucleotides 482-687 also exhibit enhancer-type activity, since constructs comprising this region (p206LtpW1) exhibited higher levels of expression than the full length regulatory region;

iv) the fragment comprising nucleotides 1-214 comprises silencer-type elements as constructs comprising this region (e.g. p687LtpW1) result in lower levels of expression compared with the levels of expression obtained with either of the truncated regulatory region constructs, p206LtpW1, or p473LtpW1 (see Table 3);

v) the 206 bp version of the LtpW1 regulatory region represents a novel, constitutive promoter, for monocotyledonous plants

Because of the relatively superior activity of the 473 bp fragment in aleurone tissue (Tables 1, 2 and 3), this version was used for transformation of maize.

Example 4: Preparation of transgenic plants of *Zea mays*

To verify that the 5' flanking sequence from the genomic clone LtpW1 contained the regulatory sequences required to confer expression in aleurone cells, the 473 bp LtpW1 /GUS fusion was co-bombarded with a bialaphos selectable plasmid (pAHC25) into embryogenic cultures of maize. Transgenic calli were selected on bialaphos media and transgenic plants regenerated. The transgenic plants were screened for GUS activity. The 473 bp LtpW1 regulatory region directed the expression of GUS only in the aleurone layer of developing and germinating transgenic maize kernels (Figure 6).

Example 5: Stable expression of a gene of interest in monocots under the control of p206LptW1 (TAP).

Several lines of corn were transformed via particle bombardment as described above with a p206Ltp-OXO construct (Fig. 10) which generates oxalate oxidase reporter activity (Byron Lane et al., J.Biol. Chem.1991, 266:10461-10469). This construct also contains an intron, for example the IVS6 intron, or the first intron from the actin gene. Plants were also transformed with oxalate oxidase under the control of the strong monocot rice actin promoter (McElroy D., Zhang, W., Cao, J., and Wu, R. 1990. Plant Cell 2:163-171). Plant tissues were harvested and assayed for oxalate oxidase activity. Plants comprising the oxalate oxidase transgene under the control of p206LtpW1 exhibited substantially higher activity than the non-transformed CK44 line. Line 13-1-5 expressed at 95% of the level observed in a line homozygous for the oxalate oxidase transgene, under the control of the rice actin promoter (Fig.10).

15

Example 6: Activity of p206Lpt (TAP) in dicotyledons

The activity of the 206 bp *Hin*II/*Bcl*II truncated regulatory element (TAP; SEQ ID NO3; Figure 3) was also examined within dicotyledonous plants to determine whether this regulatory element is active within these plants. Constructs comprising GUS in operative association with TAP (p206LtpW1) were prepared as outlined in Example 3, however, an intron (either the IVS6 or actin intron) was included. These constructs were introduced into several dicotyledonous plants using particle bombardment as outlined in Example 3. A chimeric construct comprising TAP (p206LtpW1), linked with the *Bst*YI fragment of T1275, an enhancer element obtained from tobacco (see US 5,824,872, which is incorporated herein by reference), and in operative association with GUS, was also examined. The activities of the constructs comprising the p206Lpt (TAP) regulatory region was compared with the activity of the strong monocot rice actin promoter (McElroy D., Zhang, W., Cao, J., and Wu, R. 1990. Plant Cell 2:163-171). The results are present in Table 6.

30

Table 6. Activity of p-206LtpW1-GUS in leaf tissue of dicotyledonous plants and regulation by an enhancer element, *BstYI**

5	Plasmid	Gus Positive Foci per Shot		
		Soya	Brassica	Nicotiana
	Au*	1	0	0
	pAct-GUS	0	0	6
	p206LtpW1-GUS	3	123	48
	pBstY1-206LtpW1-GUS**	133	91	155

10 *Mean of three bombardments, assayed 48 hours post-bombardment.

* Au is a gold control, no added DNA.

** BstY1 is the -394 to -62 fragment of T1275 regulatory region (US 5,824,872).

15 The rice actin monocot expression vector (pAct-GUS) had no activity in *Soya* and *Brassica* and low activity in *Nicotiana* leaves. However, the 206LtpW1 regulatory region was active in *Soya*, *Brassica* and *Nicotiana* and exhibited substantially greater activity than the actin promoter in *Brassica* and *Nicotiana*.

20 These results indicate that the truncated LtpW1 regulatory region, 206LtpW1 (TAP; SEQ ID NO 3) is subject to regulation by chimeric enhancer fragments such as the *BstYI* element of T1275 (US 5,824,872). The 206LtpW1 regulatory region with the *BstYI* enhancer element had uniformly high activity in the dicotyledonous plants investigated.

25 Collectively these results indicate that the truncated regulatory region, p206Ltp, is active in a range of monocot and dicot plants, and its regulatory activity may be modulated in the presence of other exogenous regulatory elements.

Example 7: Deletion and mutational analysis of p206Lpt (TAP)

30

Preparation of constructs

A series of deletions, mutations, substitutions and additions within the TAP regulatory region were prepared to further characterized this region and its activity within both dicots and monocots.

5

1) TAP 206 deletion series:

The DNA sequences specified below were amplified by PCR and subcloned into vector p206 TAP-GUS (Figure 12(A); -206TAP). The existing restriction endonucleases *EcoRI* and *NcoI* were used to excise the 206TAP-intron region from the promoter sequence. The p206 TAP-GUS sequence was used as template for each PCR reaction to generate a deletion series TAP 150, TAP 140, TAP 130, TAP 120, TAP 110, TAP 100, and TAP 50. A GUS antisense (GUS AS) primer and matching TAP targeted sequence to which *EcoRI* and *KpnI* restriction endonucleases were added was used to generate TAP deletion sequences with an exception of TAP110 in which *EcoRI* and *Nsi* restriction sites were used. PCR product was digested with *EcoRI/NcoI* and subcloned into the vector (p206TAP).

20

206 deletion series primers: (Restriction endonuclease sites underlined)

25

TAP 150: TTT GAA TTC GGT ACC TCC ACG CAT CTC TCG CTC G (SEQ ID NO:27)
 TAP 140: TTT GAA TTC GGT ACC CGC ATC TCT CGC TCG AAC (SEQ ID NO:28)
 TAP 130: TTT GAA TTC GGT ACC GCT CGA ACC CCT ATT TAA (SEQ ID NO:29)
 TAP 120: TTT GAA TTC GGT ACC CTA TTT AAG CCC CTC CC (SEQ ID NO:30)
 TAP 110: TTT GAA TTC ATG CAT CCC TCC ATT CTT CCC TAC (SEQ ID NO:31)
 TAP 100: TTT GAA TTC GGT ACC TTC CCT ACA TTC TCC ACA CAA CC (SEQ ID NO:32)
 TAP 50: TTT GAA TTC GGT ACC ATC ACT ACG TAA TAC GGT GC (SEQ ID NO:33)

30

GUS AS: TCA CGG GTT GGG GGT TCT AC (SEQ ID NO:34)

-130 to -120/-113 to -116 mutation

This mutation was prepared as the -113 to -116 region was observed to be AT rich, therefore, this region was modified to determine the effect of this sequence on TAP activity. In order to introduce the modifications into this region, other restriction sites were introduced in the -120 to -120 region as described below.

5

The mutation was generated by PCR in two steps:

1. The first PCR primers (I) contained GUS antisense (GUS AS) and 110TAP with *EcoR1/Nsi* ends as follows:

10 5'----TT GAA TTC ATG CAT CAC TAT ATA GCC CCT CC--3' (bold = *EcoR1* and *Nsi* respectively; SEQ ID NO: 35)

2. The second primers (II) were 120TAP antisense with *Pst* end + M13 forward PCR.

15 5----'TTTT CTG CAG GGG TTC GAG CGA GAG ATG CG----3' (bold = *PstI*; SEQ ID NO: 36)

The PCR products from primers I and II were excised with *NsiI/NcoI* and *PstI/EcoRI* respectively and ligated. The resulting *EcoR1/NcoI* construct was subsequently subcloned into p206TAP digested with *Nsi/Nco*. The resultant construct comprised the dicot TATA consensus sequence along with extra nucleotides associated with the introduction of the *PstI* and *Nsi* restriction sites.

25

-120 to -80 deletions

The deletion was generated by PCR in two steps.

1. The first PCR product was generated by GUS antisense primer (GUS AS) and matching sequence of -80TAP with *Nsi* restriction endonuclease as follows:

30 5----'TTT ATG CAT ACC ACG AGT TGC TCA TCT CC--3' (bold = *NsiI*; SEQ ID NO:37)

2. The second primers were -120TAP antisense (as described above) + M13 forward PCR.

The PCR products obtained from 1 and 2, above were digested with *Nsi* and *Pst*, respectively and ligated. The resulting construct was excised with *EcoRI/Nco* and subcloned into p206TAP.

2) Preparation of new TAP regulatory region constructs

A series of mutations within the TAP nucleotide sequence were prepared (TAP 1- TAP 5; see Figures 13(A) and (C)) by PCR using primers pr1-5 and the pr6 as the primers (listed in Table 7) and the plasmid -206TAP-GUS as the template. The PCR product was digested with *NcoI* and *KpnI*, and the resulting fragment was used to replace the *NcoI* and *KpnI* fragment in -206TAP-GUS.

Chimeric TAP constructs

To generate GCC(4)TAP and G(5)TAP constructs (see Figure 13(B)), a 46-bp fragment:

AATTGCCGCCACTAGCCGCCGACCGAGCCGCCAAGAGCCGCCAGCT (SEQ ID NO:38) containing four GCC boxes (GCCGCC), or a 54-bp fragment:

AATTGCCGCCACGTGCCGCCACGTGCCGCCACGTGCCGCCACGTGCCGCCAGCT (SEQ ID NO: 39),

containing five G-boxes (GCCGCCACGT) was ligated into the *EcoRI* and *PstI* site located upstream of the -206TAP-GUS construct.

The (BST2)TAP constructs (Figure 13(B)) were generated by PCR using pr7 and pr8 (Table 7) as the primers and the -394 to -62 fragment of T1275 regulatory region (see US 5,824,872) as the template. The PCR product was digested with *EcoRI* and *SacI*, and the resulting fragment was ligated into the *EcoRI* and *PstI* site located upstream of the -206TAP-GUS construct.

Table I. Oligonucleotides used in PCR to create constructs

Primer	Sequence
5 pr1	ctaggaatcca atccttcgga agggaaaaag (SEQ ID NO:14)
pr2	agcttgggcc cgaaggatcc agggaaaaag aaaaaggggt c (SEQ ID NO:15)
pr3	agcttgggcc atccttcgga ggaatccct aaaaaggggt cctgctgcac (SEQ ID NO:16)
pr4	agcttgggcc atccttcgga agggaaaaag cccggaatccg cctgctgcac cagcgactaa (SEQ ID NO:17)
pr5	agcttgggcc atccttcgga agggaaaaag aaaaaggggt aagtggatcc cagcgactaa
10 accatccacg (SEQ ID NO:18)	
pr6	atataagcttgggggtttctacaggacg (SEQ ID NO:19)
pr7	cggaaatcgaaagcttgcctgcctgcagg (SEQ ID NO:20)
pr8	aattgagctcagcatgatcaaaaggggaaac (SEQ ID NO:21)

15

Preparation of test material and transient assay analysis

Corn type II callus was grown *in vitro* in N6 basal salt medium (Sigma USA) supplemented with 1.4 g/l proline, 0.7 g/l asparagine, 0.3g/L glutamine, 1.0 mg/l 2,4-D, B5 vitamins (Gamborg, O.L, et al. (1968), J. Exp. Res. 50, 151-158) and 2% sucrose w/v, (pH 5.8). The petri dishes were kept in dark at 22°C and the growth medium was replaced with fresh medium at interval of 3-4 weeks. The calli were transfer to fresh growth medium at least 5-7 days prior to gene transfer via particle gun delivery system.

25

Fresh callus tissue for bombardment was placed on 1cm² filter paper on callus growth medium as described above. 2.5ug of test plasmid and luciferase DNA was precipitated onto gold microprojectiles using 25uL microprojectile solution (1.78mg of 1uM gold in 25 ul glycerol), 25uL of 2.5M calcium chloride and 10uL of 100mM spermidine, rinsed with ethanol and re-suspended in 40uL of 100% ethanol. 10uL of solution was spotted onto the center of each macrocarrier and used to shoot callus

30

tissue using 1 100 psi rupture disks in the PDS-1000 BioRad Gene Gun. The callus was kept in dark at 22 C for 48hours prior to GUS assay.

To assay GUS activity the callus was harvested and frozen in liquid nitrogen.

5 The tissue was ground in extraction buffer containing of 100 mM Potassium phosphate (pH 7.8), 1 mM 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid, 10% glycerol, 0.5% Triton X-100 and 7 mM 2-mercaptoethanol. The extract was homogenized for 1 minute using a hand held homogenizor and centrifuged for 15 min at 4°C and the supernatant was used for GUS assays.

10

For fluorometric GUS assays (Jefferson, R.A. (1987) Plant Mol. Biol. Rep. 5: 387-405), 170 μ L of the crude extract was incubated at 37°C with 1 mM 4-methylumbelliferyl glucuronide in 0.3 mL of GUS assay buffer (50 mM NaPO₄, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 10 mM β -mercaptoethanol). After 0,

15 0.5, 1 and 2 h of incubation, 0.1-mL aliquots were removed and added to 1.9 mL of 0.2 M Na₂CO₃ to terminate the reaction. GUS activity was expressed as picomoles of 4-methylumbelliferone per milligram of protein per minute.

For histochemical GUS assay, tissue was incubated in a 0.5 mg/ml solution of

20 5-bromo-4-chloro-indolyl β -D-glucuronide in 100 mM sodium phosphate buffer, pH 7.0, infiltrated in a vacuum for half an hour and incubated at 37°C overnight. Following the incubation, tissue was washed in 70% ethanol.

For luciferase assays, 20 μ l of cell extract was placed in a luminometer cuvette,

25 and then 200 μ l of luciferase assay buffer (25 mM Tricine, pH 7.8, 15 mM MgCl₂, 5 mM ATP, and 1 mg/ml BSA) was added. The mixture was allowed to equilibrate to room temperature (about 15 min). Placement of the cuvette in the counting chamber of a luminometer automatically activated the machine and 100 μ l of 500 μ M luciferin was injected into the cuvette to start the reaction. The emitted

30 photons were integrated over a 10-s period and expressed as relative light units/10 s. Correction for differences in sample variability and transfection efficiency was done

by normalization of the GUS activity with luciferase activity in the light unit, yielding the GUS to luciferase ratio of each sample.

Wheat callus production was induced by placing 14 - 20 day old embryos of the variety SuMais 3, embryo side down on callus induction Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with 2,4-D, (Weeks J.T. et al. (1993). Pl. Phys. 102:1077-1084; Weeks, J.T. (1995), Stable transformation of wheat by microprojectile bombardment. In: Gene transfer to plants. Eds. I Potrykus and G. Spangenberg. Springer. pp. 157-161). When significant callus production was observed within 5 to 31 days these were crushed onto filter paper, 4 embryos per plate and transferred to fresh media in preparation for bombardment (Harvey, A., L. et al. (1990), Plant Cell Tissue and Organ Culture. 57:153-156).

Tobacco leaves were harvested from *in vitro* cultures maintained on MS medium (Murashige, T and F. Skoog. 1962. Physiol. Plant. 15:473-497). Leaves selected for bombardment were of uniform size and colour which were then preincubated on MS medium containing NAA and BA overnight prior to bombardment.

Construct DNA was extracted and purified, and each sample was diluted to a concentration of 1 $\mu\text{g}/\mu\text{l}$ for use in the bombardment protocol. Prior to bombardment tungsten particles were coated with transforming DNA by adding the following chilled sterile solutions in order 5 μL DNA, 25 μL 2.5 M CaCl_2 and 5 μL spermidine. Wheat callus and tobacco leaf tissue was bombarded with 2 μL of the DNA/tungsten solution. The settings were 100 psi pressure for wheat and 150 psi pressure for tobacco with the tissue sitting at position 10 in the particle bombardment device (Brown *et al.* 1994, Buckley *et al.* 1995).

Tissue was incubated in a growth chamber overnight following bombardment.

For histochemical analysis the tissue was covered with 3 mL GUS incubation buffer and left overnight in the dark at 37°C. Visual counts were made of positive GUS staining using a dissecting microscope.

5 For fluorometric analysis tissue was collected and ground in liquid nitrogen and either stored at -80°C or immediately extracted following protocols modified from Gartland et al. (Gartland, M.A., et. al. (1995), Fluorometric GUS analysis for transformed plant material. In: Methods in Molecular Biology, Vol. 44: Agrobacterium Protocols. Eds: K.M.A. Gartland and M.R. Davey Humana Press Inc., Totowa NY. 10 pp 195-199) and Vitha et al. (Vitha, S. K et. al. (1993), *Biologia Plantarum* 35(1):151-155). Fluorometric readings were taken on a RF-Mini 150 Recording Fluorometer, and protein content was assessed using a Bradford assay read in the BioRad Model 2550 EIA Reader. Fluorometric data was analysed using Lotus 123 and Microsoft Excel.

15 Results from transient expression analysis of the constructs shown in Figure 12(A) in corn callus is shown in Figure 12(B). Deletions from -206 to about -110 of the TAP regulatory region did not have any appreciable effect of the activity of the p206Ltp regulatory region. However, the region between -100 and -110 (see GUS 20 activity associated with the -110 TAP and -100 TAP constructs) was essential for high expression levels since deletion of this region resulted in a dramatic decrease in regulatory region activity as indicated by GUS analysis. Similar results were observed using a histochemical analysis of bombarded corn callus, where deletion of -100 to -50 region resulted in no regulatory region activity (data not shown).

25 Transient analysis of the -150 TAP, -100 TAP and -50 TAP deletion constructs in wheat culture is shown in Figure 14(A), and in tobacco culture is shown in Figure 14(C). Analysis of the -150 TAP construct in wheat, along with two other mutations, -130 to -120/ -113 to -116 mutation, and the -120 to -80 deletion construct are shown 30 in Figure 14 (B). In wheat, high levels of regulatory region activity are still observed with the -150 TAP construct (Figure 14(B)), while there is a dramatic decreased in the

activity of the TAP regulatory element with the removal of the -150 region as indicated with the activity associated with the -100 TAP construct compared with the -150 TAP construct (Figure 14(A)), or the -120 to -80 region (Figure 14(B)), suggesting that key regulatory elements reside within this region (i.e. the -150 to -100- region). The -113 to -116 region is AT rich region, and substituting several bases within this region (as in the case of the -130 to -120/ -113 to -116 mutation construct; see above), resulted in a decrease in activity (Figure 14(B)). These results collectively indicate that the -150 to -100 region is required for TAP regulatory region activity in cereals, for example wheat and corn.

10

Similar analysis of the deletion constructs in tobacco culture demonstrate that a significant amount of activity is associated with the -150 TAP, -100 TAP and the -50 TAP constructs, and that there is no loss of activity with the removal of the -150 to -100, or -50 region. These data suggests that the sequence from -50, which includes the intron (see Figure 12(A); the intron used in these constructs is the ADH 156 intron), exhibits regulatory region activity in dicots.

Analysis of the mutation constructs disclosed in Figures 13 (A) and (B) in wheat is shown in Figure 14(A). The TAP 1 to TAP 4 constructs all exhibit a large increase in activity when compared with the activity associated with the p206Ltp (TAP) construct. Similarly, chimeric regulatory elements comprising TAP and G(5), or (Bst)2 also exhibit an increase in regulatory region activity (Figure 14(A)). Collectively these results demonstrate that substitutions or additions to the Ltp regulatory region, preferably the p206Ltp region, result in increased activity when determined with a monocot plant.

25

Analysis of the mutation constructs disclosed in Figures 13 (A) and (B) in tobacco is shown in Figure 14(C). The TAP 1 to TAP 5 constructs exhibit a significant and similar level of regulatory region activity. Chimeric regulatory elements comprising (Bst)2 TAP, GCC (4) TAP or G(5) TAP also exhibit significant regulatory region activity (Figure 14(C)). Collectively these results demonstrate that

30

substitutions or additions to the Ltp regulatory region, preferably the p206Ltp region, result in increased activity when determined with a monocot plant.

All citations are herein incorporated by reference.

5

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

10

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A regulatory element comprising, the nucleotide sequence of SEQ ID NO:3, or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:3 under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity.
2. The regulatory element of claim 1, wherein said regulatory element exhibits constitutive activity.
3. The regulatory element of claim 2, wherein said regulatory element is a chimeric regulatory element, comprising an exogenous regulatory element selected from the group consisting of an enhancer element and a silencer element.
4. The regulatory element of claim 3, wherein said exogenous regulatory element is an enhancer element.
5. The regulatory element of claim 4, wherein said enhancer element is selected from the group consisting of a 35S enhancer, an actin enhancer, a *BstY1* fragment of T1275, one or more GCC box elements, and one or more Gbox elements.
6. A vector comprising the regulatory element of claim 1 in operative association with a gene of interest.
7. A transformed plant cell culture comprising the vector of claim 6.
8. A transgenic plant comprising the vector of claim 6.
9. A transgenic seed comprising the vector of claim 6.

10. The transgenic plant of claim 8, wherein the plant is a monocotyledonous plant.
11. The transgenic plant of claim 8, wherein the plant is a dicotyledonous plant.
12. A transgenic dicotyledonous plant comprising a gene construct, said gene construct comprising:
 - i) the nucleotide sequence of SEQ ID NO:3, or a fragment, mutation or derivative thereof, or a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:3 under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, wherein said nucleotide sequence exhibits regulatory element activity; and
 - ii) a gene of interest in operative association with said nucleotide sequence.
13. A regulatory element comprising, a nucleotide sequence selected from the group consisting of SEQ ID NO's:5 - 11 and 22-26 , or a fragment, mutation or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity.
14. The regulatory element of claim 13, wherein said regulatory element is 687LtpW1, or a fragment or derivative thereof.
15. The regulatory element of claim 13, wherein said regulatory element is 473LtpW1 or a fragment or derivative thereof.

16. A transgenic dicotyledonous plant comprising a gene construct, said gene construct comprising:

- i) a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26 , or a fragment, mutataion or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity; and
- ii) a gene of interest in operative association with said nucleotide sequence.

17. A transgenic monocotyledonous plant comprising a gene construct, said gene construct comprising:

- i) a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26 , or a fragment, mutation or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity; and
- ii) a gene of interest in operative association with said nucleotide sequence.

18. A method of expressing a gene of interest within a plant comprising:

- i) operatively linking a gene of interest for which expression is desired with the regulatory region of claim 1 to produce a gene construct; and
- ii) introducing said gene construct into said plant and allowing for expression of said gene of interest.

19. The method of claim 18 wherein said plant is a monocotyledonous plant.

20. The method of claim 18 wherein said plant is a dicotyledonous plant.

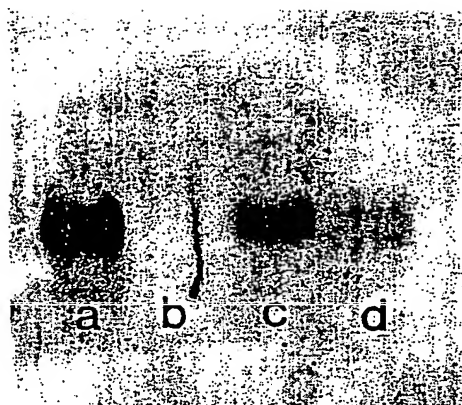


FIGURE 1

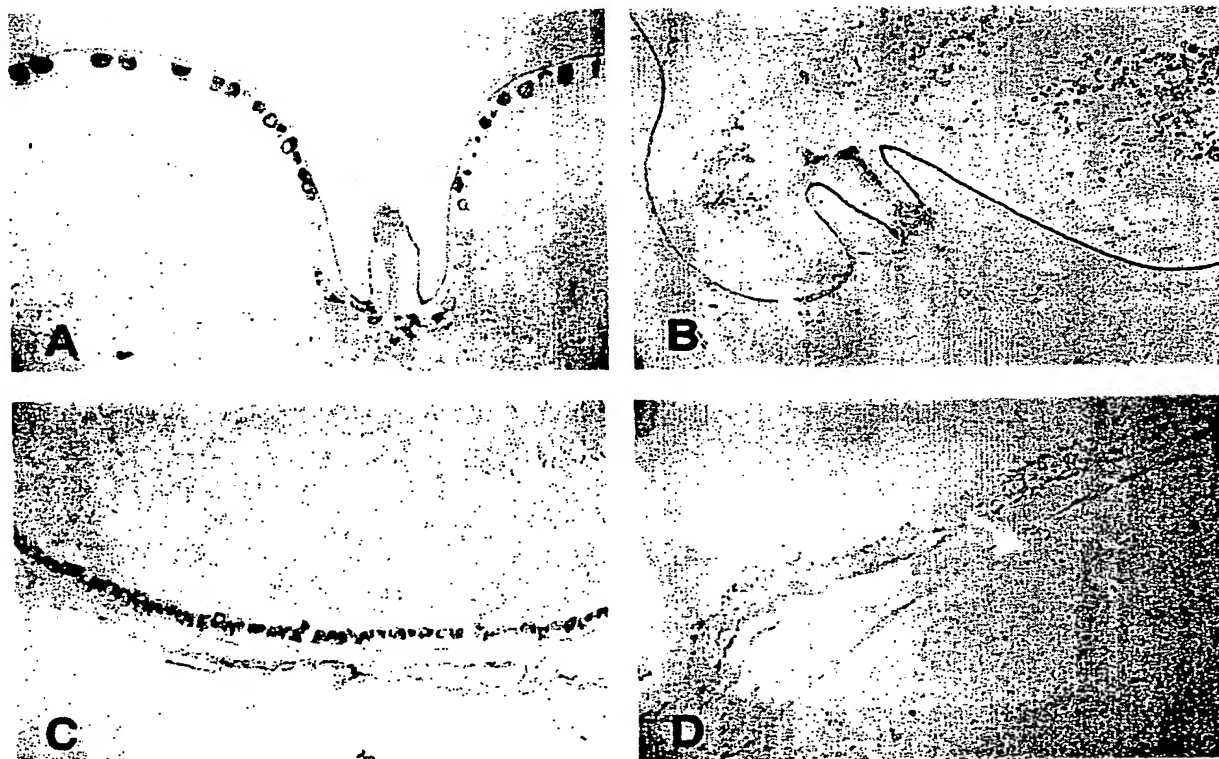


FIGURE 2

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*SEQ ID NO:1 (687 bp) >
-688 TCTAGAGAAAAGAGTTTTAGACCGGAGGTATTTGTTAGGAAGTACTTCTTGCCATACTAGT
-628 TTCAATAAAGTAGCTTGAAAAGACATTTGTTAAGCAACCATGTGTTTTTAATAATGAAGAT
-568 CCTCAATACCGAGAGCCTTTGGTCCCATGGATGACACAAAACCTCCCACTTGTTTTTTTT
*SEQ ID NO:2 (473 bp) >
-508 TTTTGTGTGTGTGTGGGTAAACTTCCCACTTGGTTAACCTATACTTCCGCTTATGTTTCA
-448 CACTTTGCCAGAAAATTGCATATGTGAAGGAAGTGCCAATATTTAATACCGTCTGGTGT
-388 ATAAATTCACTCTCCCAAAATTATTGGAGTTGAAGATTCACCTTGAAAAATAATTTGACAT
-328 ATTAAAGATGTTGCCCTTGCGCGGGGTATCTGCAAATTGAGGATCCAAGGGACGATTGCA
-268 TCCAGTTCTAAACACACCATTATGATTTTCAGTGATAATGCATGCTTCCAAAGCCAGCTG
*SEQ ID NO:3 (206 bp) >
-208 CAAGCTTGGGCCATCCTTCGGAAGGGAAAAAGAAAAAGGGGTCTGCTGCACCAGCGACT
-148 AAACCATCCACGCATCTCTCGCTCGAACCCTTATTAGCCCTCCATTCTTCCCTACAT
-88 TCTCCACACAAACCACGAGTTGCTCACTCTCTCCACCCAATCATCACTAGCTAATACGGTGC
*+1
-28 ACTGTTAGCTACAGACCAAGAAGTGATCATGCCCCGCGCTCAGGTAATGCTCATGGCCGT
33 CGCCTTGGTGCTCATGCTCGCGGCGGTCCCGCGCGCTGCCGTGGCCATCGACTGCGGCCA
93 CGTTGACAGCTTGGTGAGACCCTGCCTGAGCTACGTTAGGGCGGGCCCCGGCCCTCTGG
153 GCAGTGCTGCGACGGCGTCAAGAACCCTCCATAACCAGGCGCGATCCCAGAGCGATCGCCA
213 AAGCGCTTGCAACTGCCTCAAGGGGATCGCTCGTGGCATCCACAATCTCAACGAGGACAA
273 CGCCCGCAGCATCCCCCCCCAAGTGCGGTGTCAACCTCCCATACCATCAGTCTCAACAT
333 CGACTGCAGCAGGTGATTAATTCACATGCAAGCATATATATATGAACACTCATCCACGTA
393 AAATTTATGATATTAACATTAATCAAATCTTTGCACTGCAGGGTGTAATGGGCGACGAT
453 CCGTCAAGCTGGTGCTCAGCTCATCCATCCACGTGGAGTTGAAGCGCGCAGCCTCTATCC
513 CTATGTAGTATGGTCACTAGTTATGCGAGTTTATACTGAATATGAATAAGAACTCTCTCC
573 AGCTGGCTTGCTGGTACTCCTCTGGAGGAGATCAGTATCTGTGTACCTGAGAGTTGAGAG
633 TTGTACCATGGGCACTCCCAGTGTTTATGGACTTTAATACATACAACTCGTTCTGTTC
693 GCGTGTGACTTATCTTTGTTTCCTCACGTTGCGCTGTCATATACTCCTTCCATCCGGTAT
753 TAGTTGGCGTTCAAACGGATATATCTAGA

FIGURE 3

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[illegible]

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62 CGCGCGCTGCCGTGGCCATCGACTGCGGGCCACGTTGACAGCTTGGTGAGACCCTGCCTGA
|||||
62 CGCGCGCTGCCGTGGCCCTCAACTGCGGGCCAGGTTGACAGCAAGATGAAACCTTGCCTGA
122 GCTACGTTCAAGGGCGGCCCCGGCCCCGTCTGGGCAGTGCTGCGACGGCGTCAAGAACCTCC
|||||
122 CCTACGTTCAAGGGCGGCCCCGGCCCCGTCCGGCGAATGCTGCAACGGCGTCAGGGATCTCC
182 ATAACCAGGCGCGATCCCAGAGCGATCGCCAAAGCGCTTGCAACTGCCTCAAGGGGATCG
|||||
182 ATAACCAGGCGCAATCCTCGGGCGACCGCCAAACCGTTTGCAACTGCCTGAAGGGGATCG
242 CTCGTGGCATCCACAATCTCAACGAGGACAACGCCCCGACGATCCCCCCCCAAGTGCGGTG
|||||
242 CTCGCGGCATCCACAATCTCAACCTCAACAACGCCGCCAGCATCCCCTCCAAGTGCAATG
302 TCAACCTCCCATAACCATCAGTCTCAACATCGACTGCAGCAGGTGATTAATTCACATGC
|||||
302 TCAACGTCCCATAACCATCAGCCCCGACATCGACTGCTCCAGGTGATTAATTTACACT
362 AAGCATA.....TATATATGAAC
|||
363 CATCCAGAGTGAAATCTTTAAAAAGAACTATATTTACGAACGGAGTGAGTATATAGGAAC
380 ACTCATCCACGTAAAATTTATTGATATTAACATTAATCAAATCTTTGCA.CTGACGGGTG
|
423 ATTCATCCACGTAAAATTTGTTGATATTAACATTAACACGCATGATTGACCTGCAGGATT
440 TAATGGGCGACGATCCGTCAAGCTGGTGCTCAGCTCATCCATCCACGTGGAGTTGAAGCG
||
483 TACTGAGCGACGATCCGTCAAGCTGGTGCTCAGCTCATCGATCCACGTGGAGCTGAAGCG
500 CGCAGCCTCTATCCCTATGTAGTATGGTCACTAGTTATG.CGAGTTTATACTGAATATGA
|||||
543 CGCAGCCTCTGTCCCTATGTAGTATGGCTACCAGTTATGCCGAGTTTATGCTGA.....
559 ATAAGAACTCTCTCCAGCTGGCTTGCTGGTACTCCTCTGGAGGAGATCAGTATCTGTGTA
|||||
597 ATAAGAACTCTCTCCT.....GTACTCCTTTGGAGGAGATCAGTATCTATGTA
619 CCTGAGAGTTGAGAGTTTGTACCATGGGCACTCCCAGTGTTTATGGACTT
|
645 CGTGAGAGTTGAGAGTTTGTACCATCGGCACTCCCAGTGTTTATGGACTA

FIGURE 4a1

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-681 AAAGAGTTTTAGACCGGAGGTATTTGTTAGGAAGTACTTCTTGCCATACTAGT..TTCAA  
|| | | | |  
-822 AACCGTGGCCTAAAAATAAGCCGATGAGGATAAATAAAATGTGGTGGTACAGTACTTCAA  
  
-623 TAAAGTAGCTTGAAAAGACATTTGTTAAGCAACCATTGTGTTTTTAATATGAAGATCCTCA  
| | | || | | | | | | | | | |  
-762 GAGGTTTACTCATCAAGAGGATGCTTTTCCGATGAGCTCTAGTAGTACATCGGACCTCAC  
  
-563 ATACCGAGAGCCTTTGGTCCCATTGGATGACACAAAACCTTCCCACTTGTTTTTTTTTTTTGTG  
|||| | | | | | | | | | | |  
-702 ATACCTCCATTGTGGTGAAATATTTGTGCTCATTTAGTGATGGGTAAATTTTGTATTATG  
  
-503 TGTGTGT..GTGGGTAAACTTCCCACTTGGTTAACCTATACTTCCGCTTATGTTTCATCAC  
| | | | | | | | | | | | | |  
-642 TCACTCTAGGTTTTTGACATTTTCAGTTTTGCCACTCTTAGGTTTTGACAAATAATTTCCAT  
  
-445 TTGCCC....AGAAAATTGCATATGTGAAGGAAGTGCCAATATTTAATACCGTCTGGTG  
| | | | | | | | | | | | | |  
-582 TCCGCGGCCAAAAGCAAACAATTTTATTTTACTTTTACCACCTCTTAGCTTTCACAATGTA  
  
-390 TTATAAATTCATCTCCCAAATTATTGGAGTTGAAGATTCACCTTGAAAAATAAATTTGAC  
| | |||| | || | |||| | | | | | | | | | |  
-522 TCACAAATGCCACTCTAGAAATTC.TGTTTATGCCACAGAATGTGAAAAAAAAACACTCAC  
  
-330 ATATTA.....AAGATGTTGCCCTTGCGCGGGGTATCTGCAAATTGAGGATCCAAGGGA  
|||| | || | || | | | | | | | | | | |  
-463 TTATTTGAAGCCAAGGTGTTTCATGGCATGGAAATGTGACATAAAGTAACGTTCTGTGTATA  
  
-276 CGATTGCATCCAGT...TCTAAACACACCATTATGATTTTCAGTGATAATGCATGCTTCCA  
|| || | |||| | | | | | | | | | | |  
-403 AGAAAAAATTGTACTCCTCGTAACAAGAGACGGAAACATCATGAGACAATCGCGTTTGGG  
*SEQ ID NO:3 >  
-219 AAGCCCAGCTGCAAGCTTGGGCCATCCTTCGGAAGGGGAAAAAGAAAAGGGGTCTGCTG  
| | | | | | | | | | | | | | | | | | |  
-343 AGGCTTTGCATCACCTTTGGATGATGCGCATGAATGG.....AGTCGTCTGCTTG  
  
-159 CACCAGCGACTAAACCATCCACGCATCTCTCGCTCGAACCCCTATTTAAGCCCCCTCCATT  
| | | || | || | | | | | | | | | | |  
-293 CTAGCCTTCGCCTACCGCCCACTGAGTCCGGGCGGCAACTACCATCGGCGAACGACCCAG  
  
-99 CTTCCCTACATTCTCCACACAACCACGAGTTGCTCATCTCTCCACCCAATCATCACTAG.  
|| || | | | | | | | | | | | | | |  
-233 CTGACCTCTACCGACCGGACTTGAATGCGCTACCTTCGTGAGCGACGATGGCCGCGTACG  
.....  
  
-173 CTGGCGACGTGCCCCCGCATGCATGGCGGCACATGGCGAGCTCAGACCGTGCGTGGCTGG  
  
-40 .....CTAATACGGTGCACTGTAGCTA  
| | | | | | |  
-113 CTACAAATACGTACCCCGTGAGTGCCCTAGCTAGAACTTACACCTGCAACTGCGAGAGC
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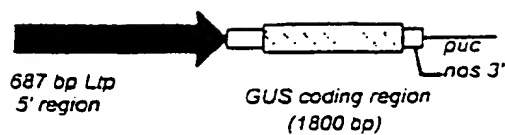
-17 CAGACCAAGAAGTGATCATG
 || || ||
-53 GAGCGTGTGA.GTGTAGCCGAGTAGATCACCGTACGACGACGACGAGGGGCATG

FIGURE 4b1

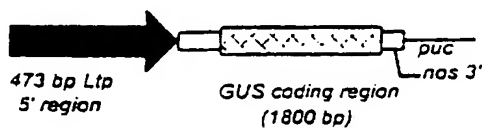
SUBSTITUTE SHEET (RULE 26)

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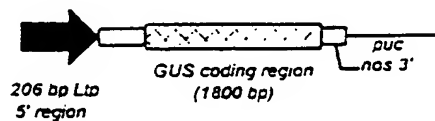
a) p687LtpW1-GUS



b) p473LtpW1-GUS



c) p206LtpW1-GUS



In all three constructs, the ADH1S6 intron lies between The LtpW1 promoter and the GUS coding region.

d) pLC-GUS

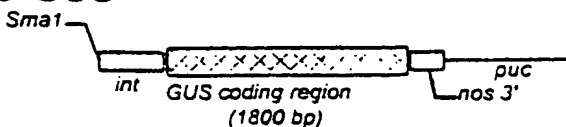
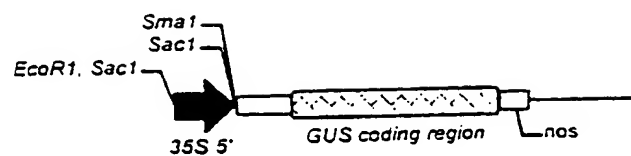


FIGURE 5

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a) p35S-GUS



The ADH1S intron lies between the promoter and the GUS gene

b) pACT-GUS



FIGURE 6

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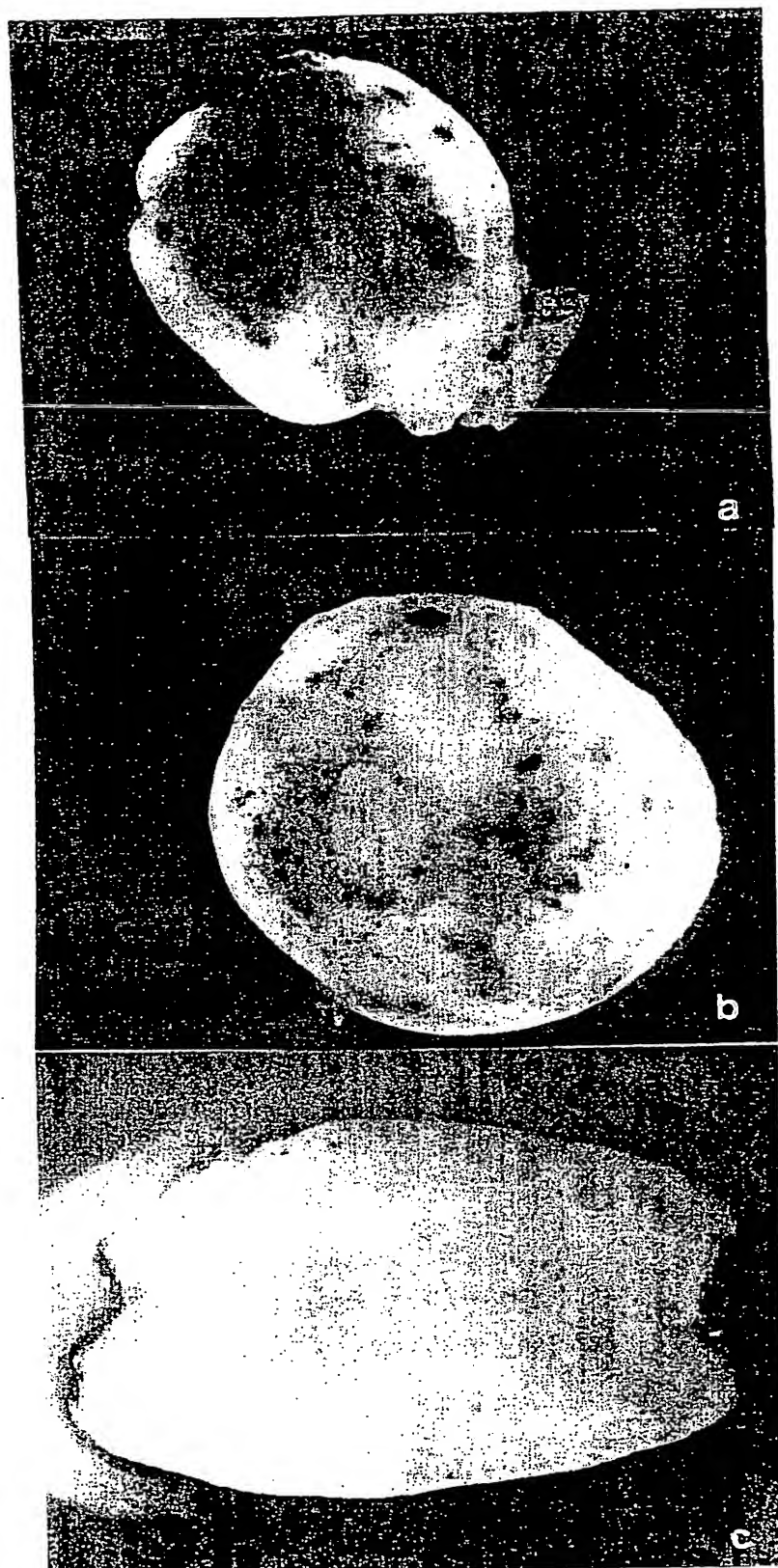


FIGURE 7

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FIGURE 8

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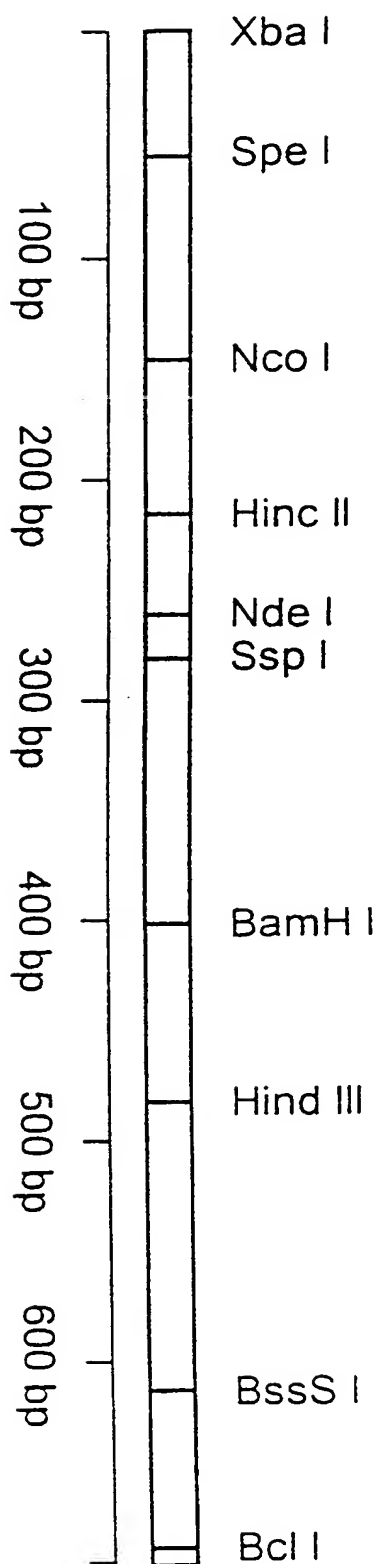


FIGURE 9

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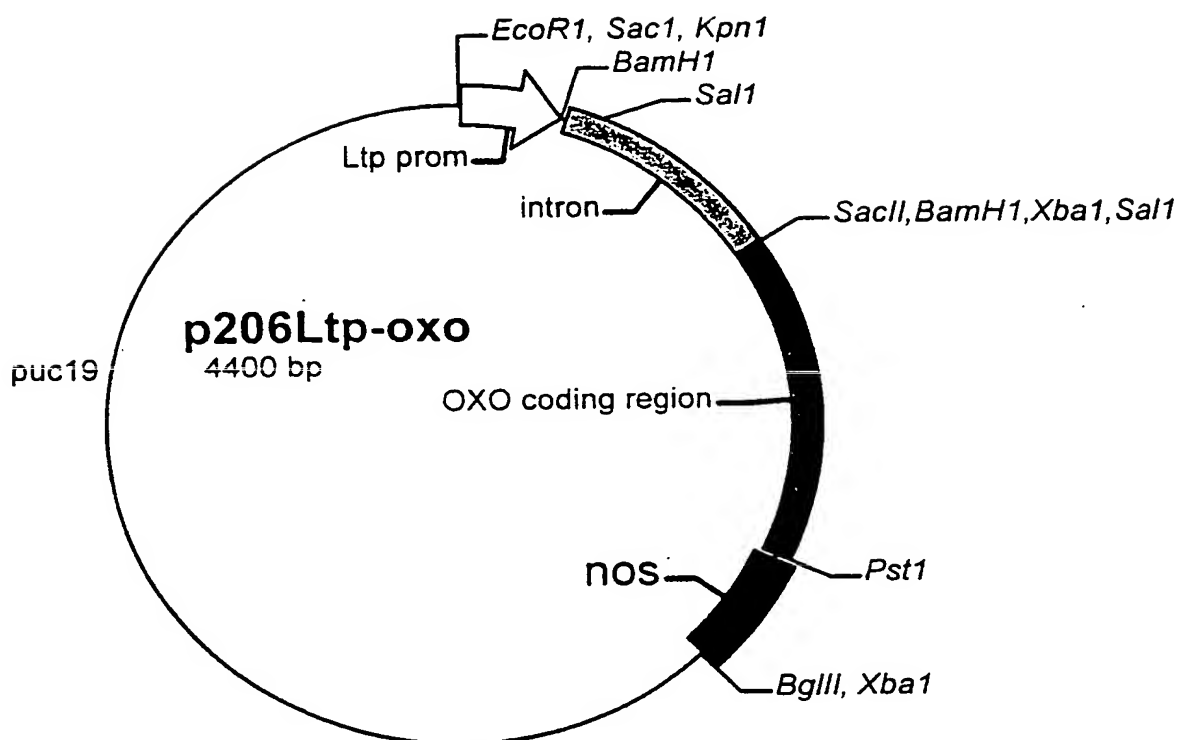


FIGURE 10

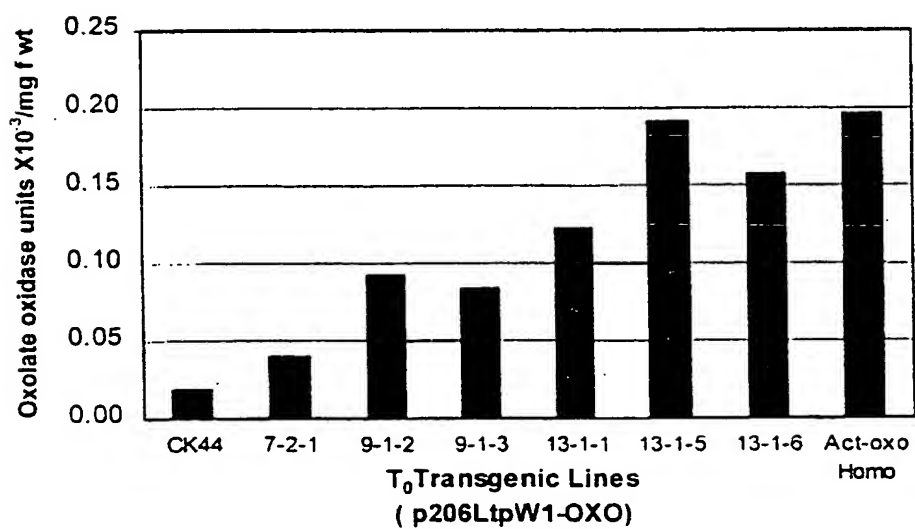


FIGURE 11

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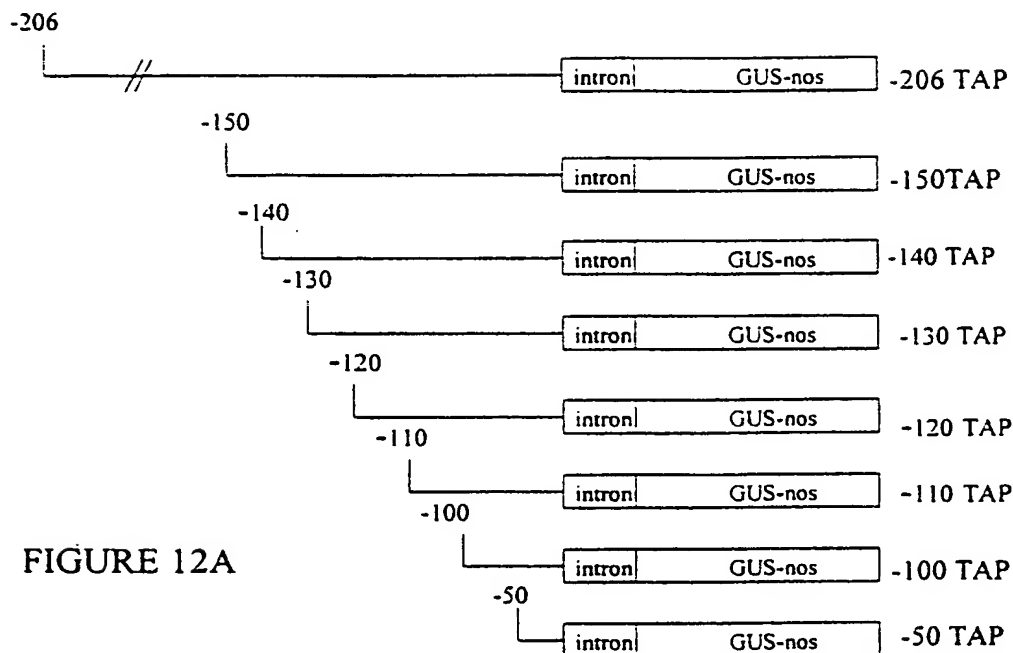
TAP 5' Deletion Series

FIGURE 12A

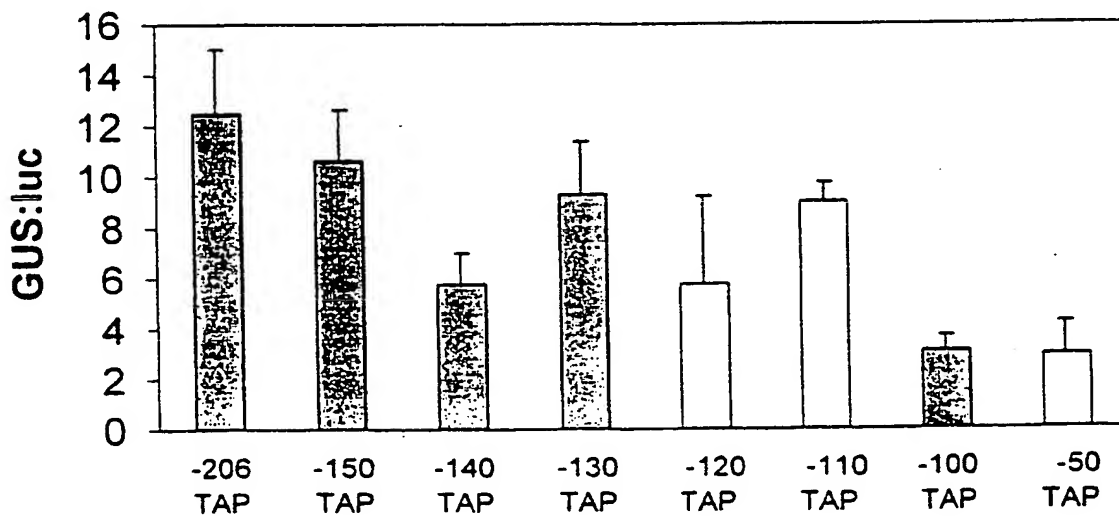
GUS expression of TAP deletion series in corn callus

FIGURE 12B

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FIGURE 13A

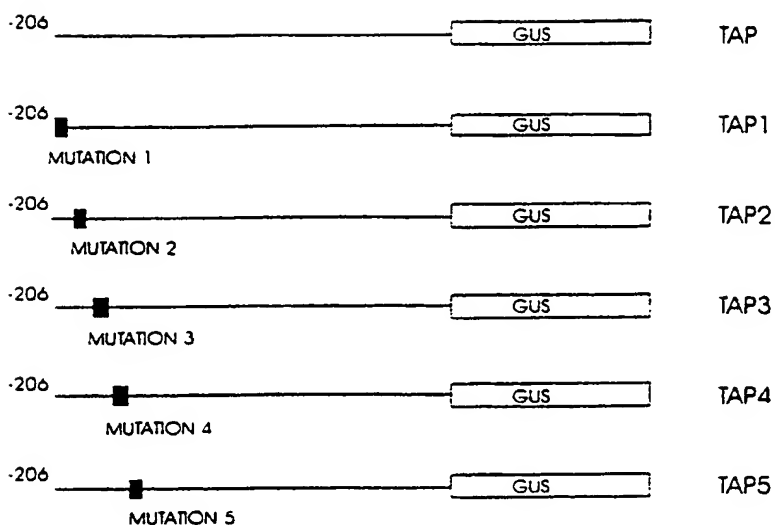


FIGURE 13B

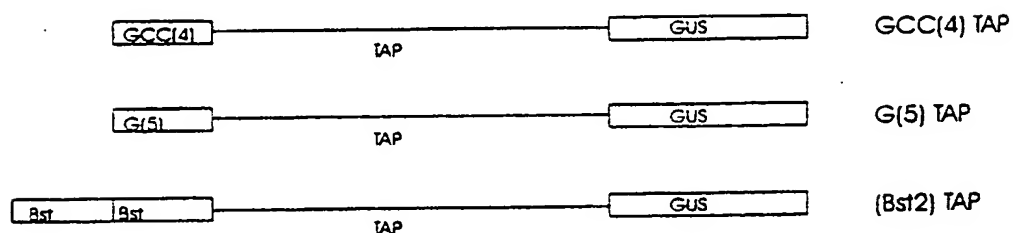
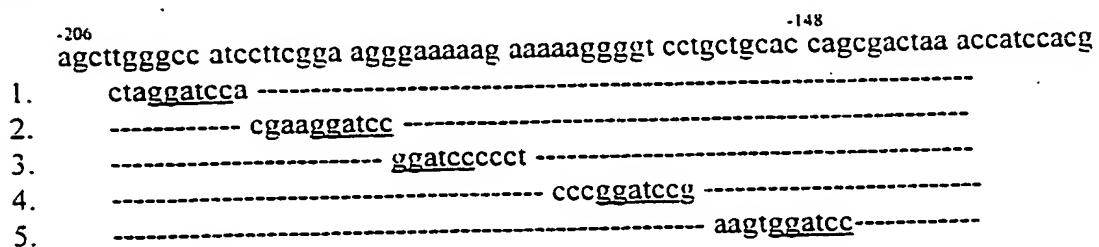


FIGURE 13C



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206 TAP.seq

caagcttggg ccaccccttcg gaagggaaaa agaaaaaggg gtcctgctgc accagcgact
 aaaccatcca cgcattctctc gctcgaaccc ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gctcatctct ccaccaatc atcactagct aatacgggtgc
 actgtttagct acagaccaag aagtgate

150 TAP

aaaccatcca cgcattctctc gctcgaaccc ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gctcatctct ccaccaatc atcactagct aatacgggtgc
 actgtttagct acagaccaag aagtgate

140 TAP

cgcattctctc gctcgaaccc ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gctcatctct ccaccaatc atcactagct aatacgggtgc
 actgtttagct acagaccaag aagtgate

130 TAP

gctcgaaccc ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gctcatctct ccaccaatc atcactagct aatacgggtgc
 actgtttagct acagaccaag aagtgate

120 TAP

ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gctcatctct ccaccaatc atcactagct aatacgggtgc
 actgtttagct acagaccaag aagtgate

110 TAP

cctccattc ttcctacat
 tctccacaca accacgagtt gctcatctct ccaccaatc atcactagct aatacgggtgc
 actgtttagct acagaccaag aagtgate

100 TAP

ttcctacat
 tctccacaca accacgagtt gctcatctct ccaccaatc atcactagct aatacgggtgc
 actgtttagct acagaccaag aagtgate

50 TAP

atcactagct aatacgggtgc actgtttagct acagaccaag aagtgate

150 (-120 to -80 deletion)

aaaccatcca cgcattctctc gctcgaaccc TGCAT accacgagtt gctcatctct
 ccaccaatc atcactagct aatacgggtgc actgtttagct acagaccaag aagtgate

150 (-130 to -120 / 113 -116 mutation)

aaaccatcca cgcattctctc gctcgaaccc TGCATCA ctatAtaGgc cctccattc
 ttcctacat tctccacaca accacgagtt gctcatctct ccaccaatc atcactagct
 aatacgggtgc actgtttagct acagaccaag aagtgate

Note: underline represents a PstI/NsiI fusion site, bold is mutations

FIGURE 13D

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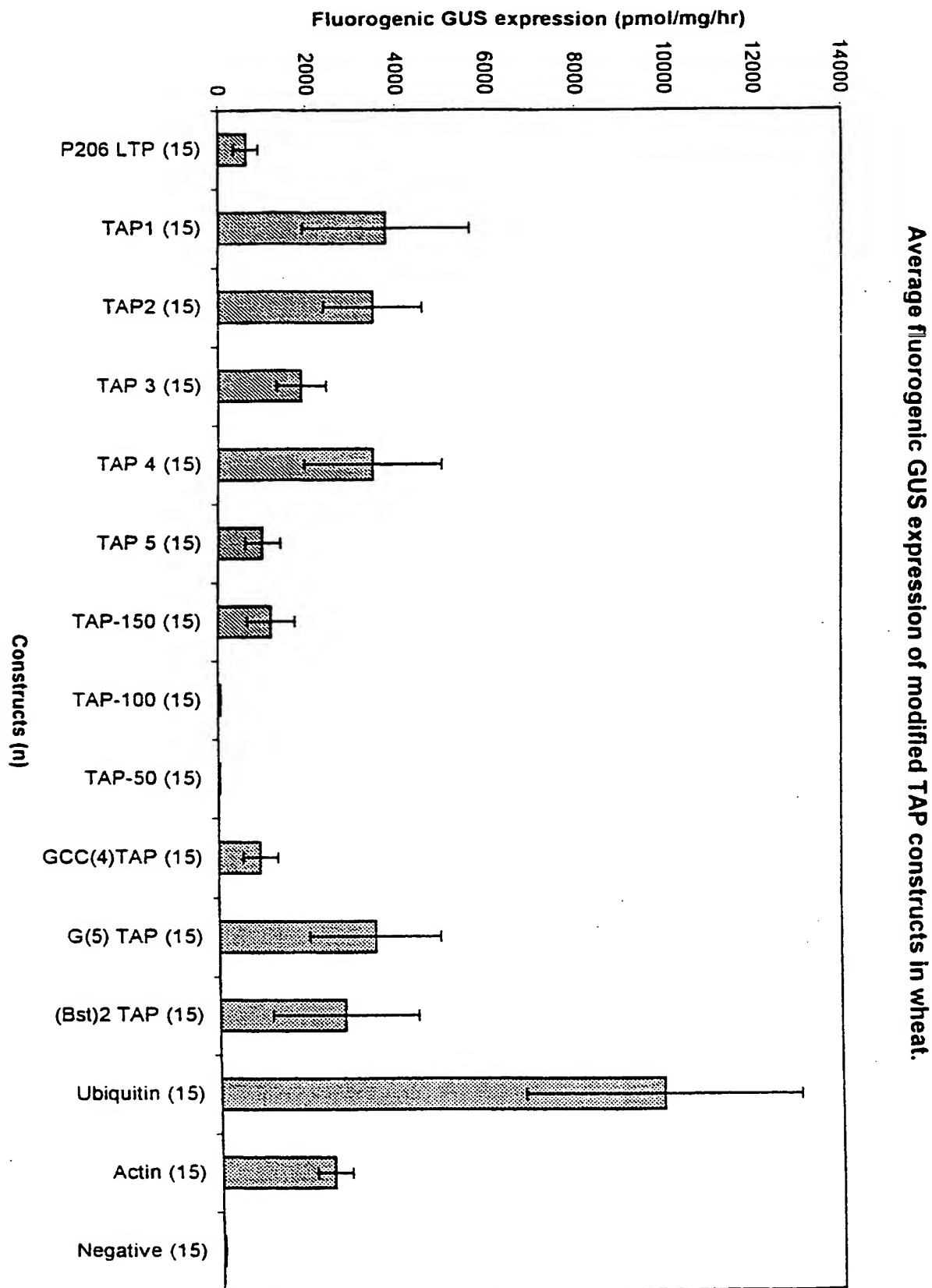


FIGURE 14A

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Mutations/Deletions in -150 TAP

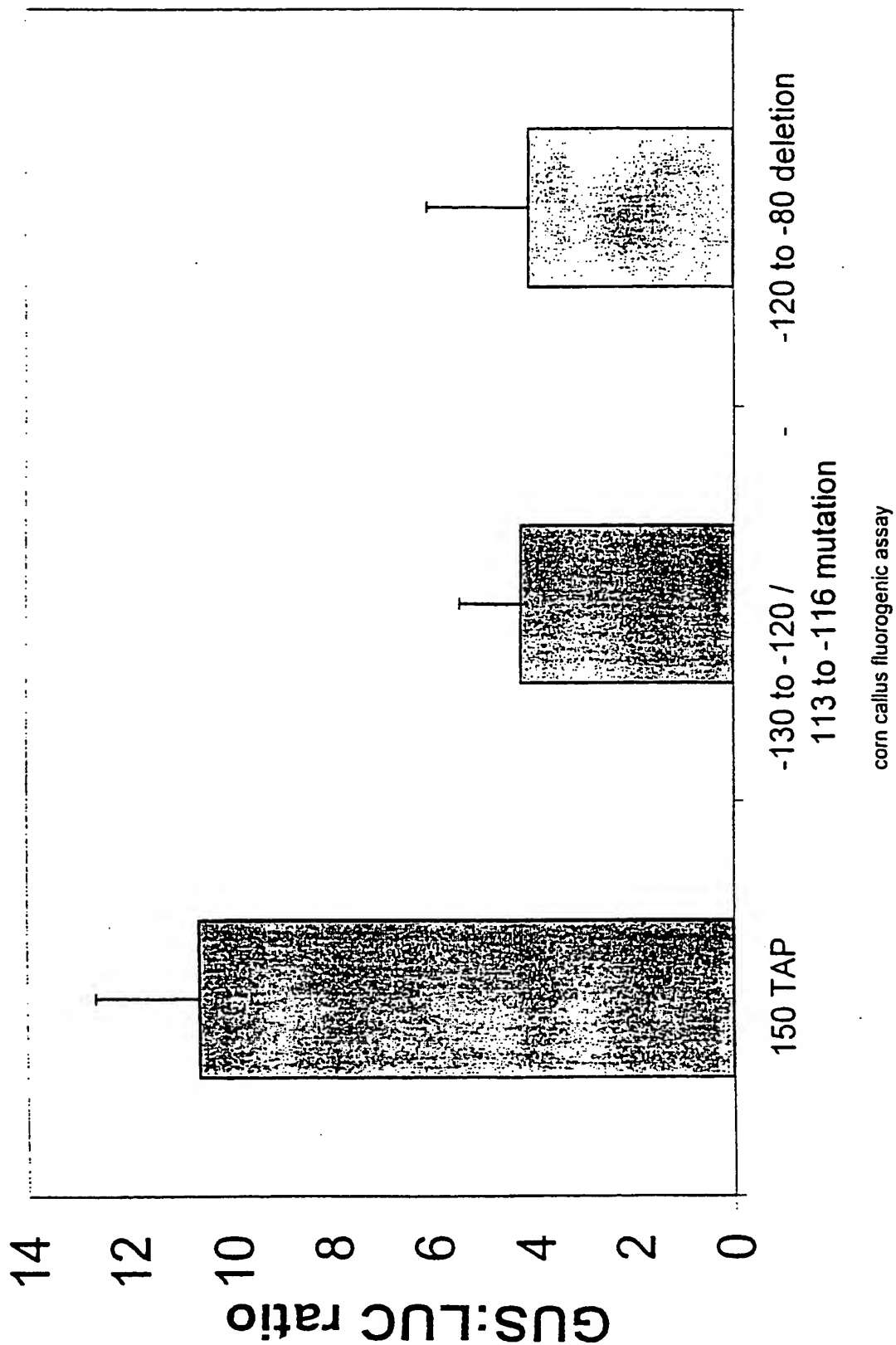


FIGURE 14B

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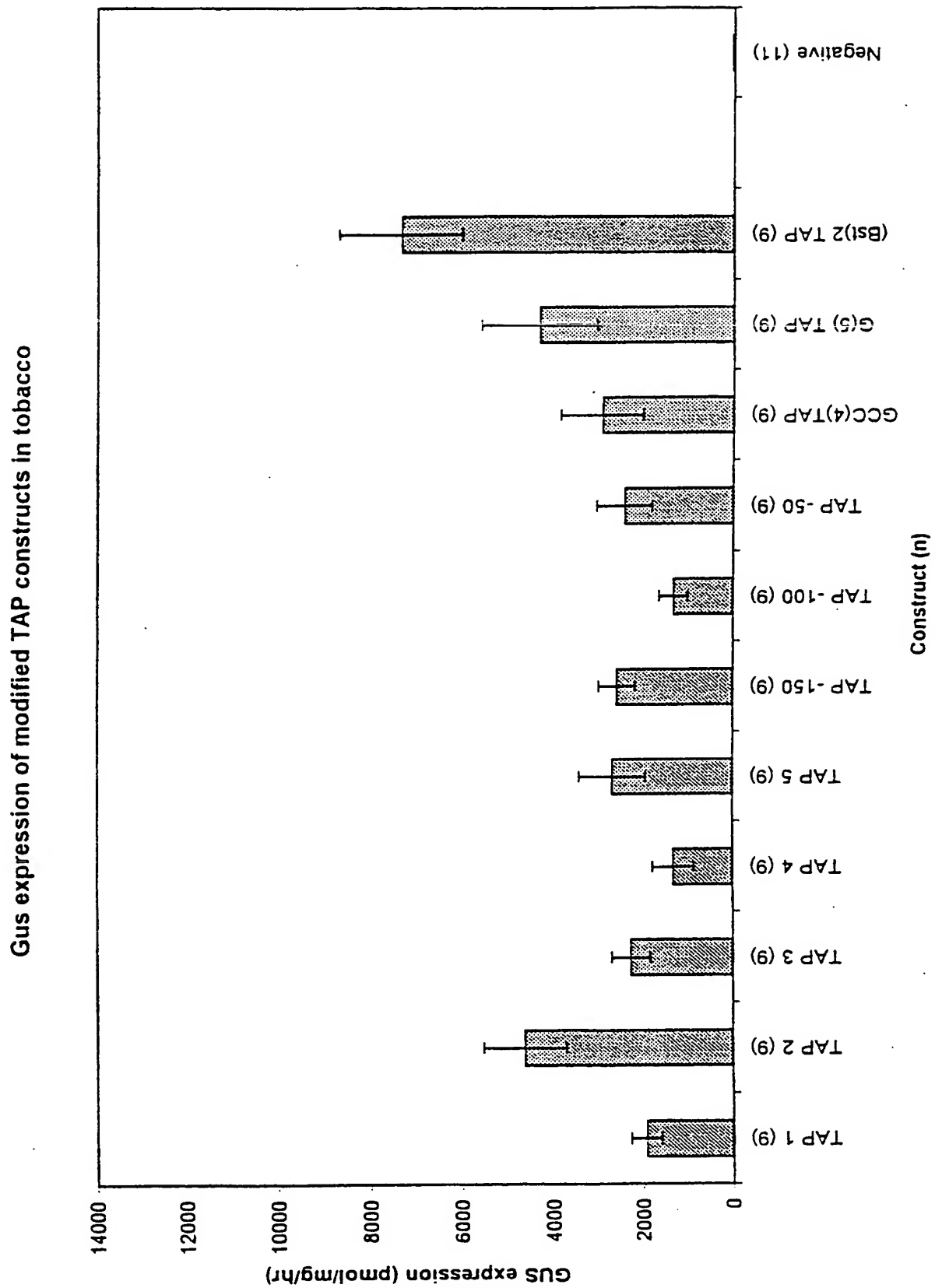


FIGURE 14C
SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: John Simmonds
(B) STREET: 130 Amberwood Cres.
(C) CITY: Nepean
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K2E 7H8

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(B) STREET: 170 Grey Fox Dr.
(C) CITY: Carp
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K0A 1L0

(A) NAME: Linda Harris
(B) STREET: 6495 Wheatfield Cres.
(C) CITY: Greely
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K4P 1E8

(A) NAME: Sharon Allard
(B) STREET: 39 Wigan Dr.
(C) CITY: Nepean
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K2E 6K9

(ii) TITLE OF INVENTION: Wheat Aleurone Regulatory region

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 687 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: LtpW1

(ix) FEATURE:

(A) NAME/KEY: promoter
(B) LOCATION: 1..687

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTAGAGAAAG AGTTTTAGAC CGGAGGTATT TGTTAGGAAG TACTTCTTGC CATACTAGTT	60
TCAATAAAGT AGCTTGAAAA GACATTTGTT AAGCAACCAT GTGTTTTTAA TATGAAGATC	120
CTCAATACCG AGAGCCTTTG GTCCCATGGA TGACACAAAA CTTCCCCTT GTTTTTTTTT	180
TTTGTGTGTG TGTGGGTAAA CTTCCCCTT GGTAAACCTA TACTTCCGCT TATGTTTCATC	240
ACTTTGCCAG AAAATTGCAT ATGTGAAGGA AGTGCCAATA TTTAATACCG TCTGGTGTTA	300
TAAATTCATC TCCCAAAATT ATTGGAGTTG AAGATTCCTT TGAAAAATA ATTTGACATA	360
TTAAAGATGT TGCCCTTGCG CGGGGTATCT GCAAATTGAG GATCCAAGGG ACGATTGCAT	420
CCAGTTCTAA ACACACCATT ATGATTTTCTG TGATAATGCA TGCTTCCAAA GCCCAGCTGC	480
AAGCTTGGGC CATCCTTCGG AAGGGAAAAA GAAAAAGGGG TCCTGCTGCA CCAGCGACTA	540
AACCATCCAC GCATCTCTCG CTCGAACCCC TATTTAAGCC CCTCCATTCT TCCCTACATT	600
CTCCACACAA CCACGAGTTG CTCATCTCTC CACCCAATCA TCACTAGCTA ATACGGTGCA	660
CTGTTAGCTA CAGACCAAGA AGTGATC	687

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 473 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: LtpW1

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION: 1..473

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AACCTATACT TCCGCTTATG TTCATCACTT TGCCAGAAAA TTGCATATGT GAAGGAAGTG	60
CCAATATTTA ATACCGTCTG GTGTTATAAA TTCATCTCCC AAAATTATTG GAGTTGAAGA	120
TTCACTTGAA AAAATAATTT GACATATTAA AGATGTTGCC CTTGCGCGGG GTATCTGCAA	180
ATTGAGGATC CAAGGGACGA TTGCATCCAG TTCTAAACAC ACCATTATGA TTTCAGTGAT	240
AATGCATGCT TCCAAAGCCC AGCTGCAAGC TTGGGCCATC CTTCGGAAGG GAAAAAGAAA	300
AAGGGGTCCT GCTGCACCAG CGACTAAACC ATCCACGCAT CTCTCGCTCG AACCCTATT	360
TAAGCCCCCTC CATTCTTCCC TACATTCTCC ACACAACCAC GAGTTGCTCA TCTCTCCACC	420
CAATCATCAC TAGCTAATAC GGTGCACTGT TAGCTACAGA CCAAGAAGTG ATC	473

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 206 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: LtpW1

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION:1..206

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGCTTGGGCC ATCCTTCGGA AGGGAAAAAG AAAAAGGGGT CCTGCTGCAC CAGCGACTAA	60
ACCATCCACG CATCTCTCGC TCGAACCCCT ATTTAAGCCC CTCCATTCTT CCCTACATTC	120
TCCACACAAC CACGAGTTGC TCATCTCTCC ACCCAATCAT CACTAGCTAA TACGGTGCAC	180
TGTTAGCTAC AGACCAAGAA GTGATC	206

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1469 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: LtpW1

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION:1..687

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCTAGAGAAA GAGTTTTAGA CCGGAGGTAT TTGTTAGGAA GTACTTCTTG CCATACTAGT	60
TTCAATAAAG TAGCTTGAAA AGACATTTGT TAAGCAACCA TGTGTTTTTA ATATGAAGAT	120
CCTCAATACC GAGAGCCTTT GGTCCCATGG ATGACACAAA ACTTCCCCT TGTTTTTTTT	180
TTTTGTGTGT GTGTGGGTAA ACTTCCCCT TGGTTAACCT ATACTCCGC TTATGTTTCT	240
CACTTTGCCA GAAAATTGCA TATGTGAAGG AAGTGCCAAT ATTTAATACC GTCTGGTGTT	300
ATAAATTCAT CTCCCAAAAT TATTGGAGTT GAAGATTCAC TTGAAAAAAT AATTGACAT	360
ATTAAAGATG TTGCCCTTGC GCGGGGTATC TGCAAATTGA GGATCCAAGG GACGATTGCA	420
TCCAGTTCTA AACACACCAT TATGATTTCG GTGATAATGC ATGCTTCCAA AGCCCAGCTG	480

CAAGCTTGGG	CCATCCTTCG	GAAGGGAAAA	AGAAAAAGGG	GTCCTGCTGC	ACCAGCGACT	540
AAACCATCCA	CGCATCTCTC	GCTCGAACCC	CTATTTAAGC	CCCTCCATTC	TTCCCTACAT	600
TCTCCACACA	ACCACGAGTT	GCTCATCTCT	CCACCCAATC	ATCACTAGCT	AATACGGTGC	660
ACTGTTAGCT	ACAGACCAAG	AAGTGATCAT	GGCCCGCGCT	CAGGTAATGC	TCATGGCCGT	720
CGCCTTGGTG	CTCATGCTCG	CGGCGGTCCC	GCGCGCTGCC	GTGGCCATCG	ACTGCGGCCA	780
CGTTGACAGC	TTGGTGAGAC	CCTGCCTGAG	CTACGTTTCA	GGCGGCCCCG	GCCCGTCTGG	840
GCAGTGCTGC	GACGGCGTCA	AGAACCTCCA	TAACCAGGCG	CGATCCCAGA	GCGATCGCCA	900
AAGCGCTTGC	AACTGCCTCA	AGGGGATCGC	TCGTGGCATC	CACAATCTCA	ACGAGGACAA	960
CGCCCGCAGC	ATCCCCCCCC	AGTGCGGTGT	CAACCTCCCA	TACACCATCA	GTCTCAACAT	1020
CGACTGCAGC	AGGTGATTAA	TTCACATGCA	AGCATATATA	TATGAACACT	CATCCACGTA	1080
AAATTTATTG	ATATTAACAT	TAATCAAATC	TTTGCACTGC	AGGGTGTAAT	GGGCGACGAT	1140
CCGTCAAGCT	GGTGCTCAGC	TCATCCATCC	ACGTGGAGTT	GAAGCGCGCA	GCCTCTATCC	1200
CTATGTAGTA	TGGTCACTAG	TTATGCGAGT	TTATACTGAA	TATGAATAAG	AACTCTCTCC	1260
AGCTGGCTTG	CTGGTACTCC	TCTGGAGGAG	ATCAGTATCT	GTGTACCTGA	GAGTTGAGAG	1320
TTTGTACCAT	GGGCACTCCC	AGTGTTTATG	GACTTTAATA	CATACAACTC	GTTCTGTTCA	1380
GCGTGTGACT	TATCTTTGTT	TCCTCACGTT	CGCCTGTCAT	ATACTCCTTC	CATCCGGTAT	1440
TAGTTGGCGT	TCAAACGGAT	ATATCTAGA				1469

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/CA 00/01185

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N15/11 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US 6 013 862 A (ALLARD SHARON ET AL) 11 January 2000 (2000-01-11) the whole document ---	1-15
P,X	CA 2 230 975 A (CANADA MIN. AGRICULTURE AGRI-FOOD CANADA) 7 November 1999 (1999-11-07) the whole document ---	1,2,6-8, 10-15
A	WO 95 23230 A (KALLA ROGER ; OLSEN ODD ARNE (NO); LINNESTAD CASPER (NO)) 31 August 1995 (1995-08-31) cited in the application page 4, line 19 -page 9, line 28 ---	1-15
A	WO 95 15389 A (KALLA ROGER ET AL) 8 June 1995 (1995-06-08) the whole document ---	1-15
-/--		

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Date of the actual completion of the international search

7 March 2001

Date of mailing of the international search report

14/03/2001

Name and mailing address of the ISA

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NL - 2280 HV Rijswijk
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Schönwasser, D

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/01185

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DIERYCK W ET AL: "NUCLEOTIDE SEQUENCE OF A CDNA ENCODING A LIPID TRANSFER PROTEIN FROM WHEAT (TRITICUM DURUM DESF.)" PLANT MOLECULAR BIOLOGY, vol. 19, 1992, pages 707-709, XP002029148 ISSN: 0167-4412 the whole document</p>	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

Original Application No

PCT/CA 00/01185

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 6013862	A	11-01-2000	NONE	
CA 2230975	A		NONE	
WO 9523230	A	31-08-1995	AU 695526 B AU 1863695 A CA 2184082 A EP 0746623 A GB 2301364 A, B US 6031152 A	13-08-1998 11-09-1995 31-08-1995 11-12-1996 04-12-1996 29-02-2000
WO 9515389	A	08-06-1995	AU 686291 B AU 1250695 A CA 2110772 A EP 0731844 A GB 2299086 A, B US 5525716 A	05-02-1998 19-06-1995 03-06-1995 18-09-1996 25-09-1996 11-06-1996

CORRECTED VERSION

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International Bureau(43) International Publication Date
19 April 2001 (19.04.2001)

PCT

(10) International Publication Number
WO 01/027296 A1(51) International Patent Classification⁷: **C12N 15/82**,
15/11, 15/63**AND AGRI-FOOD CANADA** [CA/CA]; Eastern Cere-
real & Oilseed Research Centre, K.W. Neathy Building,
Ottawa, Ontario K1A 0C6 (CA).(21) International Application Number: **PCT/CA00/01185**

(22) International Filing Date: 13 October 2000 (13.10.2000)

(72) Inventors; and

(25) Filing Language: English

(75) **Inventors/Applicants (for US only):** **SIMMONDS, John**
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K1J 6X2 (CA). **BROWN, Dan** [CA/CA]; 116 King Street,
Ilderton, Ontario N0M 2A0 (CA). **HU, Ming** [CA/CA];
279 Knudson Drive, Kanata, Ontario K2K 2N8 (CA).
MIKI, Brian [CA/CA]; 1876 Dorset Drive, Ottawa,
Ontario K1H 5V1 (CA). **WU, Keqiang** [CA/CA]; 1195
Meadowlands Drive, Apartment 11, Nepean, Ontario K2E
6J5 (CA).

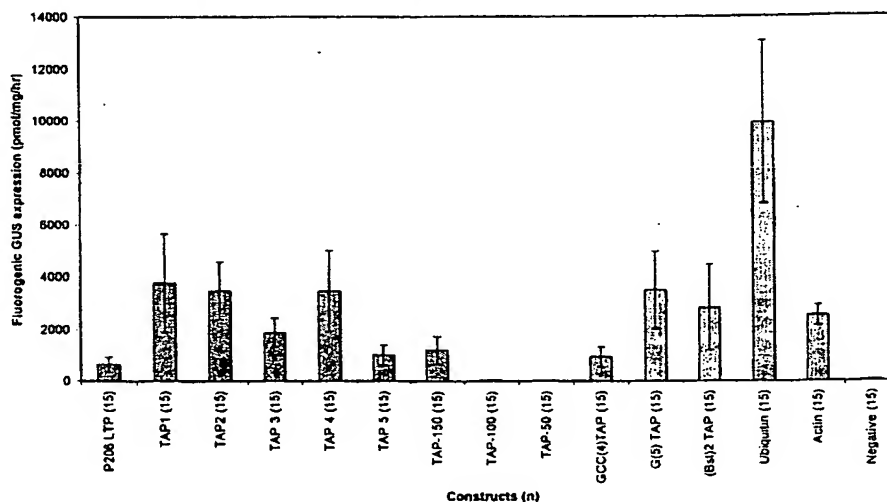
(26) Publication Language: English

(30) Priority Data:
09/417,777 14 October 1999 (14.10.1999) US(63) Related by continuation (CON) or continuation-in-part
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(54) Title: **REGULATORY REGION OF A LIPID TRANSFER PROTEIN (LTPW1) FROM ALEURON TISSUE OF WHEAT**

Average fluorogenic GUS expression of modified TAP constructs in wheat.

(57) **Abstract:** This invention is directed to a regulatory region obtained from a wheat aleurone gene LtpW1. This regulatory region, truncated derivatives, mutations, or deletions of this regulatory region, can be used to express heterologous genes of interest within aleurone cells of a plant. Furthermore, this invention is directed to a truncated LtpW1 regulatory region that exhibits constitutive activity within both monocot and dicot plants. This invention is also directed to vectors comprising these regulatory regions operatively linked with a heterologous gene of interest, as well as plant cell cultures and transgenic plants comprising these vectors. A method for the preparation of a plant using the regulatory regions of this invention are also disclosed.



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REGULATORY REGION OF A LIPID TRANSFER PROTEIN (LTPW1) FROM ALEURON TISSUE OF WHEAT

BACKGROUND OF THE INVENTION

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The present invention relates to plant gene regulatory regions and their use in the expression of genes of interest. More specifically, the present invention relates to the use of an aleurone regulatory region for organ, and tissue specific expression of a gene of interest within aleurone tissues of plants, and for constitutive expression of a gene of interest within monocot and dicotyledonous plants. This invention also pertains to derivatives of the aleurone regulatory region and their activity in monocot and dicotyledonous plants.

The endosperm of a seed is the site of deposition of storage products such as starch and proteins used by the developing embryo during germination. The endosperm surrounds the embryo of developing and mature cereal seeds. The endosperm comprises a peripheral layer of aleurone cells, which are specialized secretory cells. During germination, the aleurone layer is involved in the transfer of metabolites from the transport system to the endosperm. Furthermore, several antimicrobial compounds required to protect the seed during dormancy, imbibition and germination are synthesized within this tissue. The aleurone cells differentiate from primary endosperm cells 10-21 days after fertilization.

Several aspects of hormonal regulation of gene transcription within aleurone tissue, in germinating barley seeds have been well characterized (Fincher 1989, Annu. Rev. Plant Physiol. Mol. Biol. 40:305-346). For example, genes encoding α -amylase, responsible for the digestion of the starch stored within the starchy endosperm, and β -glucanase, which digests the cell walls, have been isolated and characterized (WO 90/01551 Rogers; US 5,677,474 Rogers, issued October 14, 1997; Karrer et al 1991, Plant Mol. Biol. 16:797-805; Slakeski and Fincher 1992). Furthermore, several structural and regulatory genes involved in anthocyanin biosynthesis within the

aleurone have been isolated and characterized (Paz-Ares et al 1987, EMBO J. 5:829-833; Dellaporta et al 1988, pp263-282 18th Stadler Genet. Symp. ed. J.P. Gustafsan and R.Appels). Other genes representing differentially expressed transcripts within aleurone layers have also been reported including CHI26 (Lea et al 1991, J. Biol. Chem. 266:1564-73); pZE40 (Smith et al 1992, Plant Mol. Biol. 20:255-66); pHvGS-1, and pcHth3 (Heck and Ho 1996, Plant Mol. Biol. 30:611-23). Several genes encoding lipid transfer proteins (Ltp) have also been obtained from barley aleurone tissues, including B11E- barley *Ltp1*, and B12A- barley *Ltp2* (Jakobsen et al 1989, Plant Mol. Biol. 12: 285-93). Only one of these genes, B12A, has been expressed ectopically in transgenic plants. In this case the promoter is active only during seed development (Kalla et al 1994 Plant J. 6:849-860)

Lipid transfer proteins are responsible for the transfer of phospholipids between membranes *in vitro*, and likely play a role during membrane biogenesis. This may be especially important in aleurone cells which are known to develop extensive membrane systems. Skriver et al (1992, Plant Mol.Biol. 18: 585-589) disclose the sequence of a genomic Ltp (*Ltp1*), including the promoter region, from barley. Northern analysis demonstrated that this gene was specifically expressed in developing and germinating seeds, as well as in whole seeds and aleurone layers obtained from seeds 30 days post anthesis (dpa). No expression of *Ltp1* mRNA was observed in root, leaf, or shoot tissues, or coleoptiles of germinating seeds. Linnestad et al (1991, Plant Physiol 97: 841-843) also discloses the promoter sequence of the *Ltp1* promoter from barley which was obtained using barley cDNA B12A as a probe. The *Ltp1* promoter, as well as a modified form of this promoter is disclosed in WO 95/23230 (February 23, 1995; Olsen et al). The modified form of the *Ltp1* promoter was not specific to directing expression within aleurone cells, and was active in a range of plant organs and tissues including aleurone cells, scutellar epithelial tissue and vascular tissue during germination or in the plant, including root, leaves and stem.

The promoter of B12A (also termed *Ltp2*) directs expression specifically within the aleurone layer of developing grain as determined using transgenic cereal plants

(Kalla et al 1994, Plant J. 6: 849-860). The sequence of the Ltp2 promoter is disclosed in CA 2,110,772 (filed December 6, 1993, Olsen and Kalla) and US 5,525,716 (Kalla et al). Dieryck et al (1992, Plt., Molec. Biol 19:707-709) disclose the incomplete cDNA sequence of a wheat (*Triticum durum*) Ltp (pTd4.90). Ltp genes comprise a multigenic family and are ubiquitous in plants. Unfortunately as these genes or corresponding proteins have been isolated from various species there is no uniformity in the terminology used to identify the genes. Hence Ltp1 from tobacco, barley and Arabidopsis are not the same. As well, two barley Ltp2 genes are described in the literature; barley Ltp2 as described in Molina and Garcia-Olmedo (Plant J. 4:983-991) is a leaf Ltp, while barley Ltp2 as described in Kalla et al (1994 Plant J. 6:849-860) is aleurone specific.

It is desirable to provide regulatory regions capable of controlling aleurone specific expression that is not detrimental to the developing embryo and seedling. Aleurone-specific regulatory regions may be used for the regulation of the expression of heterologous or native genes within aleurone tissue of cereal seeds in order to modify grain development and germination. For example, placing genes of interest under the control of aleurone-specific regulatory regions may be used to:

- 1) mediate the unloading of metabolites from the transport system into the endosperm, since this metabolite unloading is processed through aleurone cells. By expressing genes of interest involved in this process specifically within the aleurone, the grain yield may be affected. For example, which is not to be considered limiting in any manner, these genes of interest may include Na^+ and K^+ ATPases functioning in active transport, modifiers of membrane pore exclusion parameters such as TMV movement proteins, invertase for sucrose transport etc.;
- 2) affect the quality of the grain, through the production of specific proteins or enzymes, lipids, secondary metabolites etc. and their secretion into the endosperm during endosperm development or endosperm digestion. For

example, which is not to be considered limiting in any manner, such proteins may include starch synthase, ADP glucose pyrophosphorylase, monoclonal antibodies, glutenins, anticoagulants (eg hirudin), anti-pathogenic phenolics etc.. Furthermore, expression of a gene of interest within the aleurone may also be used in order to express proteins for nutritional or medicinal purposes for feeding to animals or humans;

- 3) regulate pre-harvest sprouting by affecting dormancy, for example which is not to be considered limiting, by over-expression of ACC synthase to induce inhibitory levels of ethylene;
- 4) enhance alcohol production- introduction of novel high temperature resistant enzymes for industrial application, including, but not limited to, thermostable amylases, pectinases and invertase;
- 5) modify disease resistance of developing and germinating grains by expressing proteins, for example but not limited to, oxalate oxidase, glucose oxidase, chitinase, or lipid transfer proteins, in combination with a suitable signal peptide for targeting to the extracellular matrix and cell wall localization. This approach can be used to modify the matrix to provide a stronger physical barrier against invading pathogens or to direct specific anti-pathogen agents to the aleurone/pericarp interface.

This invention characterizes a novel wheat aleurone specific regulatory region active during embryo development and germination and which controls expression of heterologous genes of interest within transgenic plants. Furthermore, this invention relates to a constitutive regulatory element that is active within monocot and dicotyledonous plants, and which can be used to drive the expression of a gene of interest in a variety of plants.

SUMMARY OF THE INVENTION

5 The present invention relates to plant gene regulatory regions and their use in the expression of genes of interest. More specifically, the present invention relates to the use of a constitutive regulatory region and derivatives of this regulatory region for expression of a gene of interest within both monocotyledonous and dicotyledonous plants.

10 Accordingly, the present invention is directed to a regulatory element comprising, the nucleotide sequence of SEQ ID NO:3, or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:3 under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity. Preferably, the regulatory element exhibits
15 constitutive activity. Furthermore, the present invention includes the regulatory element as just defined wherein the regulatory element is a chimeric regulatory element, comprising an exogenous regulatory element selected from the group consisting of an enhancer element and a silencer element.

20 This invention also pertains to a vector comprising the regulatory element defined above in operative association with a gene of interest, and to a transformed plant cell culture, a transgenic plant, either monocotyledonous or dicotyledonous plant, or a transgenic seed comprising the vector as just defined.

25 The present invention also embraces a regulatory element comprising, a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26, or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and
30 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and that exhibits regulatory element activity.

This invention relates to a transgenic dicotyledonous plant comprising a gene construct, wherein the gene construct comprises:

- 5 i) a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26 , or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and that exhibits regulatory element activity; and
- 10 ii) a gene of interest in operative association with said nucleotide sequence.

Furthermore, this invention includes a transgenic monocotyledonous plant comprising a gene construct comprising:

- 15 i) a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26 , or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and that exhibits regulatory element activity; and
- 20 ii) a gene of interest in operative association with said nucleotide sequence.

The present invention also considers a method of expressing a gene of interest within a plant comprising:

- 25 i) operatively linking a gene of interest for which expression is desired with the regulatory region of claim 1 to produce a gene construct; and
- ii) introducing said gene construct into said plant and allowing for expression of said gene of interest.

30 This method includes a plant that is a monocotyledonous plant, or a dicotyledonous plant.

5 This summary of the invention does not necessarily describe all necessary
features of the invention but that the invention may also reside in a sub-combination
of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

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FIGURE 1 shows Ltp expression in aleurone tissues of *Hordeum* and *Triticum* species using Northern analysis hybridized with a barley Ltp1 DIG-labelled cDNA. Figure 1(a) shows *Hordeum vulgare* at 20 dpa; Figure 1(b) shows *Triticum aestivum* at 10 dpa; Figure 1 (c) shows *T. aestivum* at 20 dpa; Figure 1 (d) shows *T. tungidum* at 10 dpa.

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FIGURE 2 shows RNA *in situ* hybridization of ³⁵S-labelled barley Ltp ribo-probe in 73h germinating, and 18 dpa developing wheat grain. Figure 2 (a) and Figure 2 (b) show 73h germinating wheat grain, and Figure 2 (c) and Figure 2 (d) show 18 dpa developing wheat grain. Figure 2 (a) and Figure 2 (c) show hybridization results using anti-sense probe; Figure 2 (b) and Figure 2 (d) show hybridization with sense probe.

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FIGURE 3 shows the DNA sequence of the genomic LtpW1 gene. The coding region is underlined (the intron is not underlined). The ATG start and TGG stop codon are in bold type. The cap site, TATA, CAT boxes are italicized and double-underlined at positions -83, -117 and -222, respectively. SEQ ID NO 1 runs from -687 to -1, SEQ ID NO 2 runs from -473 to -1, and SEQ ID NO 3 runs from -206 to -1.

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FIGURE 4 shows the DNA sequence alignment of LtpW1 and barley Ltp genes. Figure 4 (a) shows alignment of LtpW1 (top row) and barley Ltp1 (bottom row); Figure 4 (b) shows alignment of LtpW1 (top row) and barley Ltp2 (Kalla et al 1994 Plant J. 6:849-860). The ATG of the Ltp genes is overlined.

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- 5 **FIGURE 5** shows the LtpW1 regulatory region constructs, in all three constructs the ADH1S6 intron lies between the LtpW1 regulatory region and the coding region of the marker gene, GUS. Figure 5(a) p687LtpW1-GUS; Figure 5(b) p473LtpW1-GUS; Figure 5(c) p206LtpW1-GUS; Figure 5(d) pLC-GUS, the promoterless control used in transient assays.
- FIGURE 6** shows two constructs used for comparative studies containing prior art promoters. Figure 6(a) P35s-GUS, Figure 6(b) pACT-GUS.
- 10 **FIGURE 7** shows transient expression of LtpW1 regulatory region - GUS (p687LtpW1-GUS) fusion in aleurone of cereal grains delivered by microprojectile bombardment. Figure 7(a) shows *T. aestivum* at 15 dpa; Figure 5(b) shows *Zea mays* at 13 dpa, and Figure 7(c) shows *H. vulgare* at 12 dpa.
- 15 **FIGURE 8** shows GUS expression in aleurone layer (arrowed) of 3 days germinated kernel of *Z. mays*, T1 self progeny, transformed with p473LtpW1-GUS fusion.
- 20 **FIGURE 9** shows a restriction map of the LtpW1 regulatory region corresponding to the sequence of SEQ ID NO:1.
- FIGURE 10** shows a diagrammatic representation of the p206Ltp-oxo plasmid comprising the 206 fragment of the Ltp regulatory region (Lpt prom), adjoining an intron and the oxo coding region.
- 25 **FIGURE 11** shows the expression of p206Ltp-oxo in leaf tissue of transgenic corn. CK44 is a non-transformed control, and Act-oxo Homo is an actin-OXO T₃ line homozygous for the OXO gene, and is a high expressing positive control. The remaining T₀ transgenic lines comprise p206Ltp-oxo.
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FIGURE 12 shows a series of deletion constructs of the TAP (p206Ltp) regulatory region and there activity in corn. Figure 12(A) shows a diagrammatic representation of the 5' deletion series of the TAP regulatory region fused to the reporter GUS. Figure 12(B) shows the activity of the constructs outlined in Figure 12(A) within corn callus as determined using transient expression analysis.

FIGURE 13 shows a series of mutational TAP constructs. Figure 13 (A) shows a diagrammatical representation of a mutational series TAP 1- TAP 5. Figure 13(B) shows a diagrammatical representation of several chimeric TAP constructs, GCC(4) TAP, G(5) TAP and (Bst2) TAP. Figure 13(C) shows the substituted nucleotides within the TAP1 to TAP 5 mutational series of Figure 13(A). Substitutions 1-5 correspond to TAP 1 to TAP 5, respectively. Figure 13(D) shows the nucleic acid sequence of the deletion series for Figure 13(A).

FIGURE 14 shows activity of several regulatory regions of the present invention. Figure 14(A) shows the activity of a mutational series TAP 1 to TAP 5, and several deletion constructs, TAP-150, TAP-100, and TAP-50, and several chimeric regulatory regions, GCC(4)TAP, G(5)TAP, (Bst)2TAP in wheat callus. Figure 14(B) shows the activity of three other modified TAP regulatory regions within wheat callus. Figure 14(C) shows the activity of a mutational series TAP 1 to TAP 5, and several deletion constructs, TAP-150, TAP-100, and TAP-50, and several chimeric regulatory regions, GCC(4)TAP, G(5)TAP, (Bst)2TAP in tobacco leaf cells.

DESCRIPTION OF PREFERRED EMBODIMENT

5 The present invention relates to plant gene regulatory regions and their use in the expression of genes of interest. More specifically, the present invention relates to the use of a consitutive regulatory region for expression of a gene of interest within plants.

10 The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

Described below is a constitutive regulatory element p206LtpW1 (SEQ ID NO:3), that is active in both monocotyledonous and dicotyledonous plants. The
15 constitutive regulatory element was obtained from a wheat genomic Ltp sequence termed LtpW1 (SEQ ID NO:4). The coding region of the LtpW1 gene sequence exhibits about an 85 % homology with barley Ltp1, and includes a 26 amino acid transit peptide. The regulatory region of a wheat lipid transfer protein (Ltp) gene, LtpW1, has been isolated and characterized. This regulatory region comprises a novel
20 oligonucleotide sequence (SEQ ID NO:1), which is active in aleurone of wheat, maize and barley. The full length regulatory region is not active in leaf, root, or coleoptile tissues. However, a truncated form of LtpW1 regulatory region, termed 206Ltp, 206LtpW1 or TAP (Truncated Aleurone Promoter; SEQ ID NO 3), is active in a range of tissues and plant organs, and is active in both monocot and dicot plants.

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The regulatory region of LtpW1 compared to the barley Ltp1 regulatory region has 43 % sequence similarity with the majority of sequence similarity (82 %) occurring within 140 nucleotides upstream of the transcriptional start site (see Figure 4(a)). A minor sequence similarity was noted between LtpW1 and a barley amylase protease
30 inhibitor, however, no sequence similarity of any significance was observed between LtpW1 and Ltp2, or other known Ltp regulatory region sequences.

The full length LtpW1 regulatory region (687 nucleotides; p687LtpW1; SEQ ID NO:1), or a truncated LtpW1 regulatory region, p473LtpW1 (SEQ ID NO:2; comprising a 473 nucleotide fragment of the full length regulatory region), can be used to drive the expression of a gene of interest within the aleurone layer of a developing and germinating seed of a monocotyledonous plant, for example, but not limited to, wheat, rice, barley and maize.

LtpW1 exhibits 8.8% of 35S activity and 12.2 % activity of the strong rice action monocot constitutive regulatory region (Table 2, Example 3). Comparison of histological evidence of expression of Ltp2 (Kalla et al 1994, Plant J. 6:849-860)), with Figure 7 of the present invention (histological evidence of LtpW1 activity) indicates that LtpW1 is more than two times stronger than Ltp2.

Experiments with deletions of the LtpW1 regulatory region indicate that the 473 nucleotide fragment (SEQ ID NO: 2; p473LtpW1) of the full length regulatory region is more active in aleurone tissue than the 687 base pair regulatory region, (SEQ ID NO: 1), p687LtpW1 (Table 3). However, neither the full length regulatory region, nor the 473 bp truncated regulatory region were active in leaf tissue (see Table 4). A truncated regulatory region comprising a 206 bp nucleotide fragment (SEQ ID NO:3, p206LtpW1, also referred to as TAP) of the full length regulatory region was active in aleurone, leaf, and scutellum tissue, functioning as a minimal regulatory region element. This 206 bp region therefore represents a novel, constitutive, regulatory region useful for expressing a gene of interest within plants.

The 206bp region was also found to direct the expression of a gene of interest within leaf tissue of a range of dicotyledonous plants, including *Soya*, *Brassica* and *Nicotiana* (Table 6, Example 6). Chimeric constructs comprising the strong monocot promoter, the rice actin promoter (McElroy D., Zhang, W., Cao, J., and Wu, R. 1990. Plant Cell 2:163-171) were also introduced into these plants, and no activity was observed. These results indicate that the 206bp region also represents a novel constitutive regulatory region active in a range of plants, including both dicotyledonous

and monocotyledonous plants. Deletion and mutational analysis, and other modifications of the TAP regulatory element, including the preparation of chimeric regulatory elements, indicate that the level of TAP activity can be increased in both monocot and dicot plants (example 7).

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Figure 3 shows the sequence of the LtpW1 gene comprising the regulatory element region as identified in SEQ ID NO:1. The numbering of the regulatory region in Figure 3 is from base pairs -687 to -1, while the coding region of the gene is from base pairs +1 to 753. Therefore, p687LtpW1 comprises the sequence of SEQ ID
10 NO:1 (nucleotides 1-687), which are equivalent to the sequence of base pairs -687 to -1 of Figure 3. p473LtpW1 comprises the sequence of SEQ ID NO:2, nucleotides 214-687 of SEQ ID NO:1, or bps -473 to -1 of Figure 3. p206LtpW1 comprises the sequence of SEQ ID NO:3, nucleotides 481-687 of SEQ ID NO:1, or bps -206 to -1 of Figure 3.

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By "regulatory element" or "regulatory region", it is meant a portion of nucleic acid typically, but not always, upstream of a gene, and may be comprised of either DNA or RNA, or both DNA and RNA. The regulatory elements of the present invention includes those which are capable of mediating organ specificity, or
20 controlling developmental or temporal gene activation. Furthermore, "regulatory element" includes promoter elements, core promoter elements, elements that are inducible in response to an external stimulus, elements that are activated constitutively, or elements that decrease or increase promoter activity such as negative regulatory elements or transcriptional enhancers, respectively. It is also to be understood that
25 enhancer elements may be repeated thereby further increasing the enhancing effect of an enhancer element on a regulatory region. In the context of this disclosure, the term "regulatory element" also refers to a sequence of DNA, usually, but not always, upstream (5') to the coding sequence of a structural gene, which includes sequences which control the expression of the coding region by providing the recognition for
30 RNA polymerase and/or other factors required for transcription to start at a particular site. An example of a regulatory element that provides for the recognition for RNA

polymerase or other transcriptional factors to ensure initiation at a particular site is a promoter element. A promoter element comprises a core promoter element, responsible for the initiation of transcription, as well as other regulatory elements (as listed above) that modify gene expression. It is to be understood that nucleotide sequences, located within introns, or 3' of the coding region sequence may also contribute to the regulation of expression of a coding region of interest. A regulatory element may also include those elements located downstream (3') to the site of transcription initiation, or within transcribed regions, or both. In the context of the present invention a post-transcriptional regulatory element may include elements that are active following transcription initiation, for example translational and transcriptional enhancers, translational and transcriptional repressors, and mRNA stability determinants.

The regulatory elements, or fragments thereof, of the present invention may be operatively associated with heterologous or exogenous regulatory elements or promoters in order to modulate or mediate the activity of the heterologous regulatory element. Such modulation includes enhancing or repressing transcriptional activity of the heterologous regulatory element, modulating post-transcriptional events, or both enhancing or repressing transcriptional activity of the heterologous regulatory element and modulating post-transcriptional events. For example, one or more regulatory elements, or fragments thereof, of the present invention may be operatively associated with enhancer or silencer elements, to mediate the activity of such regulatory elements within a variety of plants. Furthermore, an intron, for example, but not limited to, the IVS6 intron from maize (Callis, J., 1987, Genes Dev. 1: 1183-1200). or the maize actin intron (McElroy, D. et al., 1991. Mol. Gen. Genet. 231: 150-160), may be included for optimizing expression within monocotyledonous or dicotyledonous plants.

An "analogue" of the regulatory elements described herein include any substitution, deletion, or additions to the sequence of a regulatory element provided that the analogue maintains at least one regulatory property associated with the activity of the regulatory element. Such regulatory properties include directing expression of

a gene in operative association with the regulatory element in one or more tissues, or directing organ-specific expression, imparting tissue specificity, or a combination thereof, or temporal activity, or developmental activity, or a combination thereof, or other regulatory attributes including, constitutive activity, negative regulatory
5 elements, enhancer sequences, or sequences that affect stability of the transcription or translation complexes or stability of the transcript.

The DNA sequences of the present invention thus include the DNA sequences of SEQ ID NO: 1 to 11, and 22 to 26, the regulatory regions and fragments thereof,
10 as well as analogues of, or nucleic acid sequences comprising about 80% similarity with the nucleic acids as defined in SEQ ID NO's: 1 to 11 or 22 to 26. Analogues (as defined above), include those DNA sequences which hybridize under stringent hybridization conditions (see Maniatis *et al.*, in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982, p. 387-389) to any one of the DNA
15 sequence of SEQ ID NO: 1 to 11 and 22 to 26, provided that said sequences maintain at least one regulatory property of the activity of the regulatory element as defined herein.

An example of one such stringent hybridization conditions may be hybridization
20 in 4XSSC at 65°C, followed by washing in 0.1XSSC at 65°C for an hour. Alternatively an exemplary stringent hybridization condition could be in 50% formamide, 5XSSC at 42°C and washing in from about 0.5XSSC to about 0.2XSSC at 65°C. Analogues include those DNA sequences which hybridize to any one of the sequences of SEQ ID NO: 1 to 11 and 22 to 26 under these hybridization conditions.

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A constitutive regulatory element directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S transcript. (Odell *et al.*, 1985, *Nature*, 313: 810-812), the rice actin 1 (Zhang *et al.*, 1991, *Plant Cell*, 3: 1155-1165) and triosephosphate isomerase 1 (Xu *et al.*, 1994, *Plant Physiol.* 106: 459-467) genes, the maize ubiquitin 1 gene (Cornejo *et al.*, 1993,
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Plant Mol. Biol. 29: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995, *Plant Mol. Biol.* 29: 637-646), T1275 (U.S. 5,824,872), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995 *Plant Mol. Biol.* 29: 995-1004). The regulatory element, p206LtpW1, as described herein, is another example
5 of a constitutive regulatory element.

The present invention also includes a chimeric regulatory region comprising the p206Ltp (also referred to as TAP) regulatory region as defined by SEQ ID NO:3, or a nucleic acid that hybridizes to SEQ ID NO: 3, as defined above, a fragment of
10 p206Ltp (TAP) regulatory region, for example but not limited to, fragments as defined by SEQ ID NO's: 5 to 11, or a substituted, modified, or mutated regulatory region, for example but not limited to the regulatory regions defined in SEQ ID NO's 22-26, and one or mediators of this regulatory activity. By "mediate", it is meant the activity associated with an exogenous regulatory element that further regulates the activity of
15 p206Ltp. For example, which is not to be considered limiting in any manner, a mediator may either up regulate, down regulate the activity of TAP, and comprise one or more enhancer or silencer elements, respectively. Examples of enhancer elements are known in the art, and include, but are not limited to, enhancers active in dicotyledonous and monocotyledonous plants, the 35S enhancer, actin enhancer, and
20 the enhancer from T1275 (*Bst*I element; U.S. 5,824,872, which is incorporated herein by reference). Furthermore, chimeric regulatory elements comprising p206Ltp or an analogue or fragment thereof may also include, but are not limited to, the addition of one or more GCC boxes, one or more G boxes or other sequences that may otherwise regulate the regulatory region activity of the regulatory region (see for example Figure
25 13(B)). Regulatory region activity associated with such chimeric regulatory regions have been demonstrated in monocots (Figure 14(A)) and dicots (Figure 14 (C)). However, it is to be understood that other chimeric TAP regulatory regions may also be prepared that exhibit desirable regulatory region properties including increasing, or modulating regulatory activity.

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Nucleic acid constructs comprising a chimeric regulatory region associated with 206LtpW1 were therefore examined to determine if exogenous regulatory regions, mediators, may further regulate the activity of TAP. Transient expression assays indicate that a dicot enhancer element, for example but not limited to an enhancer
5 obtained from tobacco, is active in increasing the activity of 206LtpW1 in plants (e.g. Table 6, Example 6). Similarly, one or more GCC boxes or one or more G boxes are also able to enhance TAP activity (see Figures 14(A) and 14(C)) in both monocot and dicot plants. Therefore, the present invention is also directed to gene constructs comprising a chimeric 206LtpW1 regulatory region, comprising p206 or a fragment
10 or analogue thereof in association with at least one other exogenous regulatory region. The present invention also pertains to the use of a chimeric regulatory region as just described in operative association with a gene of interest.

Any exogenous gene, or gene of interest, can be used and manipulated
15 according to the present invention to result in the expression of the exogenous gene.

By "gene of interest" it is meant any gene that is to be expressed within a host organism. Such a gene of interest may include, but is not limited to, a gene that encodes a protein directed at improving an agronomic trait of the plant, for example
20 but not limited to improving plant defence against pathogens, or resistance to herbicides. A gene of interest may also include, but is not limited to, a gene that encodes a pharmaceutically active protein, for example growth factors, growth regulators, antibodies, antigens, their derivatives useful for immunization or vaccination and the like. Such proteins include, but are not limited to, interleukins,
25 insulin, G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof, interferons, for example, interferon- α , interferon- β , interferon- τ , blood clotting factors, for example, Factor VIII, Factor IX, or tPA or combinations thereof. A gene of interest may also encode an industrial enzyme, protein supplement, nutraceutical, or a value-added product for feed, food, or both feed and food use. Examples of such proteins include,
30 but are not limited to proteases, oxidases, phytases, chitinases, invertases, lipases, cellulases, xylanases, enzymes involved in oil biosynthesis etc.

The chimeric gene construct of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

10 Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene
15 of the present construct can therefore be used to construct chimeric genes for expression in plants.

The chimeric gene construct of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These
20 enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the
25 source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the regulatory element selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this
30 invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide for resistance to an antibiotic such

as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β -glucuronidase), or luminescence, such as luciferase are useful.

5 The data presented herein indicate that nucleotides 1-214 and 215-481 of SEQ ID NO: 1 are responsible for imparting tissue specificity within this regulatory region, since once the nucleotides 1-481 are removed from the full length regulatory region, tissue specificity is lost (Table 4). It is contemplated that either of these regions may be combined with any suitable regulatory region of interest, for example, which is not
10 to be considered limiting, a minimal, constitutive, or viral promoter etc. in order to obtain aleurone-specific expression of a gene linked thereto. Both of these regions were found to comprise very low sequence similarity with other sequences present within gene sequence databases such as GenBank.

15 Furthermore, the data presented in Table 3 indicates that the region comprising nucleotides 1-481 of SEQ ID NO: 1 is responsible for regulating the strength of regulatory region activity, and includes both silencer- and enhancer-type activities. For example, the fragment comprising nucleotides 215-481 of SEQ ID NO:1 may be used as an enhancer like element as constructs comprising this region (e.g. p473LtpW1)
20 resulted in increased expression when compared with either the full length regulatory region (p687LtpW1) or the truncated regulatory region p206LtpW1 (see Table 3). Similarly, nucleotides 482-687 (of SEQ ID NO:1) also exhibit enhancer-type activity, since constructs comprising this region (p206LtpW1) exhibited higher levels of expression than the full length regulatory region. Therefore, it is contemplated that
25 nucleotides 214-481, or 482-687 of SEQ ID NO:1 may be combined with any suitable regulatory region of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc., in order to obtain both aleurone-specific expression of a gene linked thereto, as well as increased gene expression.

30 The present invention also pertains to the p687LtpW1 regulatory region comprising additional enhancer or silencer elements, or further comprising

modifications to the sequence of these regulatory regions, for example mutations, additions or substitutions as described in , but not limited to those of Figure 13(A), or (B).

5 Similarly, the fragment comprising nucleotides 1-214 comprises silencer-type elements as constructs comprising this region (e.g. p687LtpW1) result in lower levels of expression compared with the levels of expression obtained with either of the truncated regulatory region constructs, p206LtpW1, or p473LtpW1 (see Table 3). It is contemplated that nucleotides 1-214 may be combined with any suitable regulatory
10 region of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc., in order to obtain both aleurone-specific expression of a gene linked thereto, along with reduced gene expression.

 The truncated regulatory region, p473LtpW1, was used to transform maize,
15 where it was noted that this regulatory region was active only in aleurone of developing and germinating cereal grain.

 In transient assays, activity of the 206 bp *HinII*/*BclII* truncated regulatory element (SEQ ID NO3; Figure 3) in non-aleurone tissues was relatively low (7-11%)
20 compared to other constitutive promoters (see Table 4, Example 3). However, the level of stable expression in transformed plants (Fig10) was high, equalling the activity observed for the expression of a gene of interest under the control of a strong monocot regulatory element, the rice actin promoter (McElroy D., Zhang, W., Cao, J., and Wu, R. 1990. Plant Cell 2:163-171). Chimeric TAP regulatory regions were also
25 found to be active in directing the expression of a gene of interest in monocot and dicot tissues (see Figures 14 (A) and (C)) . Therefore, it is contemplated that the p206 (TAP) region may also be combined with any suitable regulatory region of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc. in order to obtain aleurone-specific expression of a gene linked thereto.

30

Deletion analysis of the TAP regulatory region (constructs outlined in Figure 12(A)) indicate that from -206 to about -110 (nucleotides 1- 98 of SEQ ID NO:3) of the TAP regulatory region did not have any appreciable effect of the activity of the p206Ltp regulatory region in monocots, for example corn. However, the region
5 between -100 and -110 (nucleotides 90 to 108 of SEQ ID NO:3) was observed to be required for high expression levels.

The -150 TAP (regulatory region defined in SEQ ID NO: 5), -100 TAP (regulatory region defined in SEQ ID NO:10) and -50 TAP (regulatory region defined
10 in SEQ ID NO:11) regulatory region in monocots is shown in Figure 14(A), and in dicots is shown in Figure 14(C). In monocots, high levels of regulatory region activity are observed with the -150 TAP construct (Figure 14(B)), while the removal of the -150 region removed any activity, as indicated with the activity associated with the -100 TAP construct compared with the -150 TAP construct (Figure 14(A)), or the -120 to
15 -80 region (regulatory region defined in SEQ ID NO:12; Figure 14(B)). These results suggest that one or more core regulatory elements reside within this region (i.e. the -150 to -100- region; nucleotides 58 to 108 of SEQ ID NO:3).

Nucleotides 89-96 of SEQ ID NO:3 are AT rich. To determine if this region
20 is involved with the activity of the TAP regulatory element, a construct was prepared comprising substitutions in this region (-130 to -120/-113 to -116 mutation construct, having a regulatory region defined in SEQ ID NO:13). This construct resulted in a decrease in activity in monocots (Figure 14(B)).

25 Analysis of the deletion constructs in dicots indicates that significant activity is associated with the -150 TAP, -100 TAP and the -50 TAP constructs (regulatory regions defined in SEQ ID NO's: 5, 10 and 11, respectively). Since there is no loss of activity with the removal of the -150 to -100, or -50 region, as was observed in monocots, this demonstrates that the sequence from -50 to -1 (nucleotides 158 to 206
30 of SEQ ID NO:3), exhibits regulatory region activity in dicots.

Several alterations and substitutions of the TAP regulatory region were also examined for regulatory region activity. Example of constructs TAP1 to TAP 5, which are not to be considered limiting in any manner, are shown in Figure 13 (A) (regulatory regions defined in SEQ ID NO's: 22-26). The TAP 1 to TAP 4 constructs all exhibit a large increase in activity in monocots when compared with the activity associated with the p206Ltp (TAP) construct. These data demonstrate that the regulatory region activity may be enhanced through the mutation, alteration, or substitution of nucleotides within the TAP sequence. It is also to be understood that the modifications described above to the TAP regulatory region, as well as chimeric regulatory region constructs, may also be included within the full length Ltp regulatory region, including the p687LtpW1 or p473LtpW1 regulatory regions, or fragments thereof.

Analysis of the mutation constructs disclosed in Figures 13 (A) and (B) were also examined in dicots as shown in Figure 14(C). The TAP 1 to TAP 5 constructs all exhibit a similar level, or an increase in the level of regulatory region activity when compared with the activity associated with the p206Ltp (TAP) construct. Chimeric regulatory elements comprising (Bst)2 TAP, GCC (4) TAP or G(5) TAP exhibit an increase in regulatory region activity (Figure 14(C)).

Collectively these results demonstrate that substitutions or additions to the Ltp regulatory region, preferably the p206Ltp region, are functional in both monocot and dicot plants, and result in increased activity in both monocot and dicot plants.

The gene constructs of the present invention can also include other optional regulatory motifs such as enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include, for example, the enhancer region of the 35S regulatory region, as well as other enhancers obtained from other regulatory regions, and/or the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The

translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically
5 modified so as to increase translation of the mRNA.

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed
10 and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, embryo or shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known
15 methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

The constructs of the present invention can be introduced into plant cells using
20 Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geierman and Corey, *Plant Molecular Biology*, 2d Ed. (1988); and Miki and Iyer, Fundamentals of Gene Transfer in Plants. In *Plant*
25 *Metabolism*, 2d Ed. DT. Dennis, DH Turpin, DD Lefebvre, DB Layzell (eds), Addison Wesley, Langmans Ltd. London, pp. 561-579 (1997). The present invention further includes a suitable vector comprising the chimeric gene construct.

The above description is not intended to limit the claimed invention in any
30 manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

5 **Example 1: Localization of Ltp1 Expression**

In order to isolate genes which are functional in aleurone of developing and germinating wheat grain, a barley cDNA probe of an aleurone specific lipid transfer protein gene (Ltp1,) was used to indicate activity of similar genes in wheat aleurones
10 during seed development. Northern blot analyses using a DIG -labelled barley cDNA probe showed that Ltp transcripts were present in aleurone tissue 20 dpa (Figures 1 (a) and 1(c)). No activity was detected in early wheat grain development, 10 dpa (Figure 1(b) but could be detected in *T. turgidum* (Fig. 1(d)).

15 *In situ* hybridization (based on a modification of the procedure outlined in Cox and Goldberg, 1998, Analysis of Plant Gene Expression, In Plant Molecular Biology. A Practical Approach, pp. 1-34) performed on cross sections of developing and germinating grain showed that Ltp expression was limited to aleurone cells. A ³⁵S Ltp antisense ribo-probe hybridized strongly to aleurone cells (Figure.2 (a)), whereas no
20 differential hybridization was observed with the sense RNA probe (Figure 2 (b)). Ltp expression was observed throughout grain development after 18 dpa and during germination up to 73 h post-imbibition at which time the endosperm was depleted. No hybridization was observed in developing endosperm, embryo or pericarp tissues (data not shown).

25

Example 2: Genomic DNA of LtpW1

Genomic DNA was isolated from young leaf tissue of hexaploid wheat, (*T.aestivum*) and digested with XbaI. When this DNA was analysed by Southern blot
30 using standard procedures and a DIG-labelled barley Ltp cDNA probe, three loci for the Ltp1 gene (at 1.5, 6.0, and 7.0 kb) were detected. The copy corresponding to the

1.5 kb XbaI band was cloned by screening a λ long C phage library of size-restricted XbaI fragments with a barley Ltp1 cDNA probe.

LtpW1 refers to the Ltp gene contained within the 1.5 kb XbaI digested *T. aestivum* genomic clone, the sequence of which is shown in SEQ ID NO: 4 (also see Figure 3).

The coding sequence of LtpW1 shares 85% DNA identity with the barley Ltp1 (Figure 4 (a)), includes a 26 amino acid transit peptide for cell wall localization of the protein, and has one predicted 88bp intron which is 44 bp shorter than the equivalent barley intron. The nucleotide sequences LtpW1 and barley Ltp 1 promoter (Linnestad 1991) are well conserved for approximately 140 bp upstream of the ATG start codon whereupon they diverge considerably (Figure 4 (a)). The conserved promoter region includes the putative cap and TATA sites but not the proposed CAT site or other regulatory elements (see Figures 3, and 4 (a)).

The nucleotide sequence of the LtpW1 regulatory region exhibits little or no identity with the barley Ltp2 promoter (Figure 4 (b)). The LtpW1 regulatory region was shown to be active in aleurone of developing and germinating cereal grain which is uniquely different from the barley Ltp2 promoter which is only active during grain development but not during germination (Kalla et al 1994).

Example 3: Expression of GUS under the control of LtpW1 aleurone regulatory regions.

p687LtpW1-GUS

A 687 bp XbaI/BclII regulatory region fragment (SEQ ID NO: 1; Figure3) was subcloned from pLtpW1 and fused to a GUS promoterless reporter cassette (pLC-GUS). pLC-GUS was obtained by removing the 35S promoter as a SacI fragment from pZO1016 (designated p35S-GUS herein), which was a gift from R. Sinibaldi,

Sandoz, Ca. A 687 bp XbaI/BcII fragment was isolated from pTALP1 (containing the 1.5 kb XbaI-digested *T. aestivum* genomic clone) and the sticky ends were filled-in with Klenow fragment of DNA polymerase. This fragment was blunt-end ligated into the SmaI site of pLCGUS (see Figure 5(d)), and the orientation of the insert was checked by digesting with BamHI. The activity of this regulatory region was compared with that of the promoterless construct (pLC-GUS) as well as to constructs comprising constitutive CaMV35S and rice action promoters (see Figures 6 (a) and (b), respectively). These constructs were used for comparison of promoter activities. The 35S promoter is described in: Odel, J.T., Nagy, F. and Chua N-H (1985) Nature 313:810-812. The rice actin promoter is described in: McElroy D., Zhang, W. Cao, J. and Wu, R. (1990) Plant Cell 2:163-171.

These constructs were introduced into the aleurone of cereal grains by microparticle bombardment using standard methods. LUC and GUS constructs were co-bombarded in equimolar amounts and GUS is expressed relative to LUC to minimize variability between reps (shots). LUC activity serves as an internal control for the shot to shot variability.

Tissues, 48 h post-bombardment, were incubated in reaction buffer containing 50mM NaH₂PO₄ (pH 7.0), 10 mM EDTA and 1 mM 5-bromo-4-chloro-3-indolyl-B-glucoronide (X-Gluc), 0.5mMK₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆] at 37°C for 4-20 h. A blue precipitate in the bombarded cells indicates activity of B-glucoronidase. The regulatory region gave high expression of GUS in histological transient assays with wheat aleurones (Figure 7(a)). Activity was also demonstrated in maize and barley aleurones (Figure 7(b) and (c)) The 687 bp regulatory region fragment showed no activity in leaf, root or coleoptile tissues of wheat (data not shown).

In quantitative expression assays in wheat aleurone the 687 bp regulatory region had 3.4% of the activity of the constitutive 35S promoter (Table 1). This underestimates the relative aleurone-directed activity of the LtpW1 regulatory region because of additional endosperm-derived activity of the constitutive 35S promoter.

Table 1: Activity of p687LtpW1 in 12 dpa wheat aleurone

	Construct	Luciferase (mv/sec/mg protein)	GUS (pmol MU/min/mg protein)	GUS/LUC	%35S Activity
5	Au	2001	0	0	-
	pLC-GUS ² /p35S-LUC	2100	0	0	-
	p35S-GUS/p35S-LUC	3400	30072	8.84	-
	p687LtpW1-GUS/p35S-LUC	4200	1247	0.3	3.4

10 1: mean of three sets of bombardments

2: promoterless construct

P473LtpW1-GUS

15

A truncated version of the LtpW1 regulatory region (see SEQ ID NO:2; nucleotides -473 to -1 of Figure 3; or nucleotides 214-687 of SEQ ID NO:1) was prepared by digesting pTALTP1 with HincII and BclI, and the resulting 0.47kb fragment (after treatment with Klenow) was ligated into the SmaI site of pLC-GUS.

20

Orientation of the insert was checked by digesting the resulting recombinant plasmid with BamHI. The construct (p473LtpW1-GUS) thus obtained showed 8.8% and 12.2% activity of the constitutive 35S and rice action promoters, respectively (pAct-GUS was a gift from Ray Wu at Cornell). See Table 2 for results.

Table 2: Activity of p473LtpW1 in 12 dpa wheat aleurone

	Construct	Luciferase (mv/sec/mg protein)	GUS (pmol MU/min/mg protein)	GUS/LUC	%35S Action Activity
5	Au	2001	0	0	-
	pLC-GUS ² /p35S-LUC	1300	0	0	-
	p35S-GUS/p35S-LUC	3500	8077	2.31	-
	pAct-GUS/p35S-LUC	3300	5524	1.67	
	p473LtpW1-GUS/p35S-	3200	651	0.2	8.8 12.2
10	LUC				

1: mean of three sets of bombardments

2: promoterless construct

15

When compared within a single experiment, the 473 bp fragment was 170% as active as the 687 bp fragment (Table 3).

Table 3: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in 7 dpa wheat aleurone

	Construct	Luciferase (mv/sec/ mg/protein)	GUS (pmol MU/min/mg protein)	GUS/LUC	%35S Activity
5	pLC-GUS ² /p35S-LUC	97001	120	0.01	0.1
	p35S-GUS/p35S-LUC	2300	18305	7.96	-
	p206LtpW1-GUS/p35S-LUC	7900	1781	0.23	2.9
	p473LtpW1-GUS/p35S-LUC	6800	2399	0.35	4.4
	p687LtpW1-GUS/p35S-LUC	5100	1090	0.21	2.6

10

1: mean of three sets of bombardments

2: promoterless construct

P206LtpW1-GUS

15

To generate p206LtpW1-GUS, pTALTP1 was digested with BclI, then with HindIII, and the 0.2 kb fragment was isolated from a gel and purified. The sticky ends were filled in with Klenow and the resulting fragment was ligated into the SmaI site of pLC-GUS. Neither the 687 bp or 473 bp regulatory regions was active in leaf tissue but the 206 bp HinII/BclI truncated regulatory region (SEQ ID NO:3; nucleotides -206 to -1 of Figure 3; nucleotides 481-687 of SEQ IDNO:1) had 7.5% the activity of the 35S promoter in leaf (Table 4).

20

Table 4: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in wheat leaf tissue

5	Construct	Luciferase	GUS	GUS/LUC	%35S
		(v/sec/mg/ protein)	(pmol MU/min/mg protein)		Activity
	pLC-GUS ² /p35S-LUC	2001	1.6	0.007	0.7
	p35S-GUS/p35S-LUC	200	204.3	1.02	-
	p206LtpW1-GUS/p35S-LUC	200	15.3	0.077	7.5
	p473LtpW1-GUS/p35S-LUC	700	1.3	0.002	0.2
10	p687LtpW1-GUS/p35S-LUC	700	1.7	0.002	0.2

1: mean of three sets of bombardments

2: promoterless construct

15

Similarly, in wheat scutellum tissue, only the 206 bp regulatory region fragment was active (Table 5) with activities of 11.4% of 35S and 8.5% of rice actin promoters.

Table 5: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in 20 dpa wheat scutellum tissue

5	Construct	Luciferase	GUS	GUS/LUC	%35S
		(v/sec/mg/ protein)	(pmol MU/min/mg protein)		Action Activity
	pLC-GUS ² /p35S-LUC	13001	37	0.028	0.23, 0.17
	p35S-GUS/p35S-LUC	400	4873	12.182	-
	pAct-GUS/p35S-LUC	400	6530	16.325	-
	p206LtpW1-GUS/p35S-LUC	100	139	1.39	11.41, 8.51
10	p473LtpW1-GUS/p35S-LUC	200	2	0.01	0.08, 0.06
	p687LtpW1-GUS/p35S-LUC	200	6	0.03	0.24, 0.18

1: mean of three sets of bombardments

2: promoterless construct

15

Thus the nucleotide sequence between 206 bp and 473 bp determines the tissue and stage dependent regulation of the LtpW1 regulatory region.

20 Collectively, these data indicate that:

i) nucleotides 1-214 and 215-481 of SEQ ID NO:1 are responsible for imparting tissue specificity within this regulatory region. Removal of either of these regions from the full length regulatory region results in greatly reduced tissue specificity (Table 25 4).

ii) the region comprising nucleotides 1-481 of SEQ ID NO: 1 is responsible for regulating the strength of regulatory region activity, and includes both silencer- and enhancer-type activities:

iii) the fragment comprising nucleotides 214-481 of SEQ ID NO:1 exhibits enhancer-like activity as constructs comprising this region (e.g. p473LtpW1) resulted in increased expression when compared with either the full length regulatory region (p687LtpW1), or the truncated regulatory region p206LtpW1 (see Table 3). Similarly, nucleotides 482-687 also exhibit enhancer-type activity, since constructs comprising this region (p206LtpW1) exhibited higher levels of expression than the full length regulatory region;

iv) the fragment comprising nucleotides 1-214 comprises silencer-type elements as constructs comprising this region (e.g. p687LtpW1) result in lower levels of expression compared with the levels of expression obtained with either of the truncated regulatory region constructs, p206LtpW1, or p473LtpW1 (see Table 3);

v) the 206 bp version of the LtpW1 regulatory region represents a novel, constitutive promoter, for monocotyledonous plants

Because of the relatively superior activity of the 473 bp fragment in aleurone tissue (Tables 1, 2 and 3), this version was used for transformation of maize.

Example 4: Preparation of transgenic plants of *Zea mays*

To verify that the 5' flanking sequence from the genomic clone LtpW1 contained the regulatory sequences required to confer expression in aleurone cells, the 473 bp LtpW1 /GUS fusion was co-bombarded with a bialaphos selectable plasmid (pAHC25) into embryogenic cultures of maize. Transgenic calli were selected on bialaphos media and transgenic plants regenerated. The transgenic plants were screened for GUS activity. The 473 bp LtpW1 regulatory region directed the expression of GUS only in the aleurone layer of developing and germinating transgenic maize kernels (Figure 6).

Example 5: Stable expression of a gene of interest in monocots under the control of p206LptW1 (TAP).

Several lines of corn were transformed via particle bombardment as described above with a p206Ltp-EXO construct (Fig. 10) which generates oxalate oxidase reporter activity (Byron Lane et al., J.Biol. Chem.1991, 266:10461-10469). This construct also contains an intron, for example the IVS6 intron, or the first intron from the actin gene. Plants were also transformed with oxalate oxidase under the control of the strong monocot rice actin promoter (McElroy D., Zhang, W., Cao, J., and Wu, R. 1990. Plant Cell 2:163-171). Plant tissues were harvested and assayed for oxalate oxidase activity. Plants comprising the oxalate oxidase transgene under the control of p206LtpW1 exhibited substantially higher activity than the non-transformed CK44 line. Line 13-1-5 expressed at 95% of the level observed in a line homozygous for the oxalate oxidase transgene. under the control of the rice actin promoter (Fig.10).

Example 6: Activity of p206Lpt (TAP) in dicotyledons

The activity of the 206 bp *Hin*II/*Bcl*II truncated regulatory element (TAP; SEQ ID NO3; Figure 3) was also examined within dicotyledonous plants to determine whether this regulatory element is active within these plants. Constructs comprising GUS in operative association with TAP (p206LptW1) were prepared as outlined in Example 3, however, an intron (either the IVS6 or actin intron) was included. These constructs were introduced into several dicotyledonous plants using particle bombardment as outlined in Example 3. A chimeric construct comprising TAP (p206LptW1), linked with the *Bst*YI fragment of T1275, an enhancer element obtained from tobacco (see US 5,824,872, which is incorporated herein by reference), and in operative association with GUS, was also examined. The activities of the constructs comprising the p206Lpt (TAP) regulatory region was compared with the activity of the strong monocot rice actin promoter (McElroy D., Zhang, W., Cao, J., and Wu, R. 1990. Plant Cell 2:163-171). The results are present in Table 6.

Table 6. Activity of p-206LtpW1-GUS in leaf tissue of dicotyledonous plants and regulation by an enhancer element, *BstYI**

		Gus Positive Foci per Shot		
5	Plasmid	Soya	Brassica	Nicotiana
	Au*	1	0	0
	pAct-GUS	0	0	6
	p206LtpW1-GUS	3	123	48
	pBstY1-206LtpW1-GUS**	133	91	155

10 [†]Mean of three bombardments, assayed 48 hours post-bombardment.

* Au is a gold control, no added DNA.

** BstY1 is the -394 to -62 fragment of T1275 regulatory region (US 5,824,872).

15 The rice actin monocot expression vector (pAct-GUS) had no activity in *Soya* and *Brassica* and low activity in *Nicotiana* leaves. However, the 206LtpW1 regulatory region was active in *Soya*, *Brassica* and *Nicotiana* and exhibited substantially greater activity than the actin promoter in *Brassica* and *Nicotiana*.

20 These results indicate that the truncated LtpW1 regulatory region, 206LtpW1 (TAP; SEQ ID NO 3) is subject to regulation by chimeric enhancer fragments such as the *BstYI* element of T1275 (US 5,824,872). The 206LtpW1 regulatory region with the *BstYI* enhancer element had uniformly high activity in the dicotyledonous plants investigated.

25 Collectively these results indicate that the truncated regulatory region, p206Ltp, is active in a range of monocot and dicot plants, and its regulatory activity may be modulated in the presence of other exogenous regulatory elements.

Example 7: Deletion and mutational analysis of p206Lpt (TAP)

30

Preparation of constructs

A series of deletions, mutations, substitutions and additions within the TAP regulatory region were prepared to further characterized this region and its activity within both dicots and monocots.

5

1) TAP 206 deletion series:

The DNA sequences specified below were amplified by PCR and subcloned into vector p206 TAP-GUS (Figure 12(A); -206TAP). The existing restriction endonucleases *EcoR*I and *Nco*I were used to excise the 206TAP-intron region from the promoter sequence. The p206 TAP-GUS sequence was used as template for each PCR reaction to generate a deletion series TAP 150, TAP 140, TAP 130, TAP 120, TAP 110, TAP 100, and TAP 50. A GUS antisense (GUS AS) primer and matching TAP targeted sequence to which *EcoR*I and *Kpn*I restriction endonucleases were added was used to generate TAP deletion sequences with an exception of TAP110 in which *EcoR*I and *Nsi* restriction sites were used. PCR product was digested with *EcoR*I/*Nco*I and subcloned into the vector (p206TAP).

206 deletion series primers: (Restriction endonuclease sites underlined)

20

TAP 150: TTT GAA TTC GGT ACC TCC ACG CAT CTC TCG CTC G (SEQ ID NO:27)

TAP 140: TTT GAA TTC GGT ACC CGC ATC TCT CGC TCG AAC (SEQ ID NO:28)

TAP 130: TTT GAA TTC GGT ACC GCT CGA ACC CCT ATT TAA (SEQ ID NO:29)

TAP 120: TTT GAA TTC GGT ACC CTA TTT AAG CCC CTC CC (SEQ ID NO:30)

25 TAP 110: TTT GAA TTC ATG CAT CCC TCC ATT CTT CCC TAC (SEQ ID NO:31)

TAP 100: TTT GAA TTC GGT ACC TTC CCT ACA TTC TCC ACA CAA CC (SEQ ID NO:32)

TAP 50: TTT GAA TTC GGT ACC ATC ACT ACG TAA TAC GGT GC (SEQ ID NO:33)

GUS AS: TCA CGG GTT GGG GGT TCT AC (SEQ ID NO:34)

30

-130 to -120/-113 to -116 mutation

This mutation was prepared as the -113 to -116 region was observed to be AT rich, therefore, this region was modified to determine the effect of this sequence on TAP activity. In order to introduce the modifications into this region, other restriction sites were introduced in the -120 to -120 region as described below.

5

The mutation was generated by PCR in two steps:

1. The first PCR primers (I) contained GUS antisense (GUS AS) and 110TAP with *EcoR1/Nsi* ends as follows:

10 5'----TT GAA TTC ATG CAT CAC TAT ATA GCC CCT CC---3' (bold = *EcoR1* and *Nsi* respectively; SEQ ID NO: 35)

2. The second primers (II) were 120TAP antisense with *Pst* end + M13 forward PCR.

15 5----'TTTT CTG CAG GGG TTC GAG CGA GAG ATG CG-----3' (bold = *PstI*; SEQ ID NO: 36)

20 The PCR products from primers I and II were excised with *NsiI/NcoI* and *Pst/EcoRI* respectively and ligated. The resulting *EcoR1/NcoI* construct was subsequently subcloned into p206TAP digested with *NsiI/NcoI*. The resultant construct comprised the dicot TATA consensus sequence along with extra nucleotides associated with the introduction of the *PstI* and *Nsi* restriction sites.

25 -120 to -80 deletions

The deletion was generated by PCR in two steps.

1. The first PCR product was generated by GUS antisense primer (GUS AS) and matching sequence of -80TAP with *Nsi* restriction endonuclease as follows:

30 5----'TTT ATG CAT ACC ACG AGT TGC TCA TCT CC---3' (bold = *NsiI*; SEQ ID NO:37)

2. The second primers were -120TAP antisense (as described above) + M13 forward PCR.

The PCR products obtained from 1 and 2, above were digested with Nsi and Pst, respectively and ligated. The resulting construct was excised with *EcoRI/Nco* and subcloned into p206TAP.

2) Preparation of new TAP regulatory region constructs

A series of mutations within the TAP nucleotide sequence were prepared (TAP 1- TAP 5; see Figures 13(A) and (C)) by PCR using primers pr1-5 and the pr6 as the primers (listed in Table 7) and the plasmid -206TAP-GUS as the template. The PCR product was digested with *NcoI* and *KpnI*, and the resulting fragment was used to replace the *NcoI* and *KpnI* fragment in -206TAP-GUS.

Chimeric TAP constructs

To generate GCC(4)TAP and G(5)TAP constructs (see Figure 13(B)), a 46-bp fragment:

AATTGCCGCGCCACTAGCCGCGCGACCGAGCCGCGCAAGAGCCGCGCCAGCT (SEQ ID NO:38) containing four GCC boxes (GCCGCG), or a 54-bp fragment:

AATTGCCGCGCCACGTGCCGCGCGACGTGCCGCGCGACGTGCCGCGCGACGTGCCGCGCGAGCT (SEQ ID NO: 39),

containing five G-boxes (GCCGCGCACGT) was ligated into the *EcoRI* and *PstI* site

located upstream of the -206TAP-GUS construct.

The (BST2)TAP constructs (Figure 13(B)) were generated by PCR using pr7 and pr8 (Table 7) as the primers and the -394 to -62 fragment of T1275 regulatory region (see US 5,824,872) as the template. The PCR product was digested with *EcoRI* and *SacI*, and the resulting fragment was ligated into the *EcoRI* and *PstI* site located upstream of the -206TAP-GUS construct.

Table I. Oligonucleotides used in PCR to create constructs

Primer	Sequence
5 pr1	ctaggatcca atccttcgga agggaaaaag (SEQ ID NO:14)
pr2	agcttgggcc cgaaggatcc agggaaaaag aaaaaggggt c (SEQ ID NO:15)
pr3	agcttgggcc atccttcgga ggaacccct aaaaaggggt cctgctgcac (SEQ ID NO:16)
pr4	agcttgggcc atccttcgga agggaaaaag cccggatccg cctgctgcac cagcgactaa (SEQ ID NO:17)
pr5	agcttgggcc atccttcgga agggaaaaag aaaaaggggt aagtggatcc cagcgactaa
10 accatccacg (SEQ ID NO:18)	
pr6	atataagcttgggggtttctacaggacg (SEQ ID NO:19)
pr7	cggaaatcgaaagcttgcctgcctgcagg (SEQ ID NO:20)
pr8	aattgagctcatgcatggaacaaaaggggaac (SEQ ID NO:21)

15

Preparation of test material and transient assay analysis

Corn type II callus was grown *in vitro* in N6 basal salt medium (Sigma USA) supplemented with 1.4 g/l proline, 0.7 g/l asparagine, 0.3g/L glutamine, 1.0 mg/l 2,4-D, B5 vitamins (Gamborg, O.L, et al. (1968), J. Exp. Res. 50, 151-158) and 2% sucrose w/v, (pH 5.8). The petri dishes were kept in dark at 22°C and the growth medium was replaced with fresh medium at interval of 3-4 weeks. The calli were transfer to fresh growth medium at least 5-7 days prior to gene transfer via particle gun delivery system.

25

Fresh callus tissue for bombardment was placed on 1cm² filter paper on callus growth medium as described above. 2.5ug of test plasmid and luciferase DNA was precipitated onto gold microprojectiles using 25uL microprojectile solution (1.78mg of 1uM gold in 25 ul glycerol), 25uL of 2.5M calcium chloride and 10uL of 100mM spermidine, rinsed with ethanol and re-suspended in 40uL of 100% ethanol. 10uL of solution was spotted onto the center of each macrocarrier and used to shoot callus

30

tissue using 1 100 psi rupture disks in the PDS-1000 BioRad Gene Gun. The callus was kept in dark at 22 C for 48hours prior to GUS assay.

To assay GUS activity the callus was harvested and frozen in liquid nitrogen.
5 The tissue was ground in extraction buffer containing of 100 mM Potassium phosphate (pH 7.8), 1 mM 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid, 10% glycerol, 0.5% Triton X-100 and 7 mM 2-mercaptoethanol. The extract was homogenized for 1 minute using a hand held homogenizer and centrifuged for 15 min at 4°C and the supernatant was used for GUS assays.

10

For fluorometric GUS assays (Jefferson, R.A. (1987) Plant Mol. Biol. Rep. 5: 387-405), 170 μ L of the crude extract was incubated at 37°C with 1 mM 4-methylumbelliferyl glucuronide in 0.3 mL of GUS assay buffer (50 mM NaPO₄, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 10 mM β -mercaptoethanol). After 0,
15 0.5, 1 and 2 h of incubation, 0.1-mL aliquots were removed and added to 1.9 mL of 0.2 M Na₂CO₃ to terminate the reaction. GUS activity was expressed as picomoles of 4-methylumbelliferone per milligram of protein per minute.

For histochemical GUS assay, tissue was incubated in a 0.5 mg/ml solution of
20 5-bromo-4-chloro-indolyl β -D-glucuronide in 100 mM sodium phosphate buffer, pH 7.0, infiltrated in a vacuum for half an hour and incubated at 37°C overnight. Following the incubation, tissue was washed in 70% ethanol.

For luciferase assays, 20 μ L of cell extract was placed in a luminometer cuvette,
25 and then 200 μ L of luciferase assay buffer (25 mM Tricine, pH 7.8, 15 mM MgCl₂, 5 mM ATP, and 1 mg/ml BSA) was added. The mixture was allowed to equilibrate to room temperature (about 15 min). Placement of the cuvette in the counting chamber of a luminometer automatically activated the machine and 100 μ L of 500 μ M luciferin was injected into the cuvette to start the reaction. The emitted
30 photons were integrated over a 10-s period and expressed as relative light units/10 s. Correction for differences in sample variability and transfection efficiency was done

by normalization of the GUS activity with luciferase activity in the light unit, yielding the GUS to luciferase ratio of each sample.

Wheat callus production was induced by placing 14 - 20 day old embryos of the variety SuMais 3, embryo side down on callus induction Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with 2,4-D, (Weeks J.T. et al. (1993). Pl. Phys. 102:1077-1084; Weeks, J.T. (1995), Stable transformation of wheat by microprojectile bombardment. In: Gene transfer to plants. Eds. I Potrykus and G. Spangenberg. Springer. pp. 157-161). When significant callus production was observed within 5 to 31 days these were crushed onto filter paper, 4 embryos per plate and transferred to fresh media in preparation for bombardment (Harvey, A., L. et al. (1990), Plant Cell Tissue and Organ Culture. 57:153-156).

Tobacco leaves were harvested from *in vitro* cultures maintained on MS medium (Murashige, T and F. Skoog. 1962. Physiol. Plant. 15:473-497). Leaves selected for bombardment were of uniform size and colour which were then preincubated on MS medium containing NAA and BA overnight prior to bombardment.

Construct DNA was extracted and purified, and each sample was diluted to a concentration of 1 $\mu\text{g}/\mu\text{l}$ for use in the bombardment protocol. Prior to bombardment tungsten particles were coated with transforming DNA by adding the following chilled sterile solutions in order 5 μL DNA, 25 μL 2.5 M CaCl_2 and 5 μL spermidine. Wheat callus and tobacco leaf tissue was bombarded with 2 μL of the DNA/tungsten solution. The settings were 100 psi pressure for wheat and 150 psi pressure for tobacco with the tissue sitting at position 10 in the particle bombardment device (Brown *et al.* 1994, Buckley *et al.* 1995).

Tissue was incubated in a growth chamber overnight following bombardment.

For histochemical analysis the tissue was covered with 3 mL GUS incubation buffer and left overnight in the dark at 37°C. Visual counts were made of positive GUS staining using a dissecting microscope.

5 For fluorometric analysis tissue was collected and ground in liquid nitrogen and either stored at -80°C or immediately extracted following protocols modified from Gartland et al. (Gartland, M.A., et. al. (1995), Fluorometric GUS analysis for transformed plant material. In: Methods in Molecular Biology, Vol. 44: Agrobacterium Protocols. Eds: K.M.A. Gartland and M.R. Davey Humana Press Inc., Totowa NY. 10 pp 195-199) and Vitha et al. (Vitha, S. K et. al. (1993), *Biologia Plantarum* 35(1):151-155). Fluorometric readings were taken on a RF-Mini 150 Recording Fluorometer, and protein content was assessed using a Bradford assay read in the BioRad Model 2550 EIA Reader. Fluorometric data was analysed using Lotus 123 and Microsoft Excel.

15 Results from transient expression analysis of the constructs shown in Figure 12(A) in corn callus is shown in Figure 12(B). Deletions from -206 to about -110 of the TAP regulatory region did not have any appreciable effect of the activity of the p206Ltp regulatory region. However, the region between -100 and -110 (see GUS 20 activity associated with the -110 TAP and -100 TAP constructs) was essential for high expression levels since deletion of this region resulted in a dramatic decrease in regulatory region activity as indicated by GUS analysis. Similar results were observed using a histochemical analysis of bombarded corn callus, where deletion of -100 to -50 region resulted in no regulatory region activity (data not shown).

25 Transient analysis of the -150 TAP, -100 TAP and -50 TAP deletion constructs in wheat culture is shown in Figure 14(A), and in tobacco culture is shown in Figure 14(C). Analysis of the -150 TAP construct in wheat, along with two other mutations, -130 to -120/ -113 to -116 mutation, and the -120 to -80 deletion construct are shown 30 in Figure 14 (B). In wheat, high levels of regulatory region activity are still observed with the -150 TAP construct (Figure 14(B)), while there is a dramatic decreased in the

activity of the TAP regulatory element with the removal of the -150 region as indicated with the activity associated with the -100 TAP construct compared with the -150 TAP construct (Figure 14(A)), or the -120 to -80 region (Figure 14(B)), suggesting that key regulatory elements reside within this region (i.e. the -150 to -100- region). The -113 to -116 region is AT rich region, and substituting several bases within this region (as in the case of the -130 to -120/ -113 to -116 mutation construct; see above), resulted in a decrease in activity (Figure 14(B)). These results collectively indicate that the -150 to -100 region is required for TAP regulatory region activity in cereals, for example wheat and corn.

10

Similar analysis of the deletion constructs in tobacco culture demonstrate that a significant amount of activity is associated with the -150 TAP, -100 TAP and the -50 TAP constructs, and that there is no loss of activity with the removal of the -150 to -100, or -50 region. These data suggests that the sequence from -50, which includes the intron (see Figure 12(A); the intron used in these constructs is the ADH 156 intron), exhibits regulatory region activity in dicots.

15

Analysis of the mutation constructs disclosed in Figures 13 (A) and (B) in wheat is shown in Figure 14(A). The TAP 1 to TAP 4 constructs all exhibit a large increase in activity when compared with the activity associated with the p206Ltp (TAP) construct. Similarly, chimeric regulatory elements comprising TAP and G(5), or (Bst)2 also exhibit an increase in regulatory region activity (Figure 14(A)). Collectively these results demonstrate that substitutions or additions to the Ltp regulatory region, preferably the p206Ltp region, result in increased activity when determined with a monocot plant.

20

25

Analysis of the mutation constructs disclosed in Figures 13 (A) and (B) in tobacco is shown in Figure 14(C). The TAP 1 to TAP 5 constructs exhibit a significant and similar level of regulatory region activity. Chimeric regulatory elements comprising (Bst)2 TAP, GCC (4) TAP or G(5) TAP also exhibit significant regulatory region activity (Figure 14(C)). Collectively these results demonstrate that

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substitutions or additions to the Ltp regulatory region, preferably the p206Ltp region, result in increased activity when determined with a monocot plant.

All citations are herein incorporated by reference.

5

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

10

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A regulatory element comprising, the nucleotide sequence of SEQ ID NO:3, or a fragment, mutation, or derivative thereof, or a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:3 under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity.
2. The regulatory element of claim 1, wherein said regulatory element exhibits constitutive activity.
3. The regulatory element of claim 2, wherein said regulatory element is a chimeric regulatory element, comprising an exogenous regulatory element selected from the group consisting of an enhancer element and a silencer element.
4. The regulatory element of claim 3, wherein said exogenous regulatory element is an enhancer element.
5. The regulatory element of claim 4, wherein said enhancer element is selected from the group consisting of a 35S enhancer, an actin enhancer, a *BstY1* fragment of T1275, one or more GCC box elements, and one or more Gbox elements.
6. A vector comprising the regulatory element of claim 1 in operative association with a gene of interest.
7. A transformed plant cell culture comprising the vector of claim 6.
8. A transgenic plant comprising the vector of claim 6.
9. A transgenic seed comprising the vector of claim 6.

10. The transgenic plant of claim 8, wherein the plant is a monocotyledonous plant.
11. The transgenic plant of claim 8, wherein the plant is a dicotyledonous plant.
12. A transgenic dicotyledonous plant comprising a gene construct, said gene construct comprising:
 - i) the nucleotide sequence of SEQ ID NO:3, or a fragment, mutation or derivative thereof, or a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:3 under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, wherein said nucleotide sequence exhibits regulatory element activity; and
 - ii) a gene of interest in operative association with said nucleotide sequence.
13. A regulatory element comprising, a nucleotide sequence selected from the group consisting of SEQ ID NO's:5 - 11 and 22-26 , or a fragment, mutation or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity.
14. The regulatory element of claim 13, wherein said regulatory element is 687LtpW1, or a fragment or derivative thereof.
15. The regulatory element of claim 13, wherein said regulatory element is 473LtpW1 or a fragment or derivative thereof.

16. A transgenic dicotyledonous plant comprising a gene construct, said gene construct comprising:

- i) a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26 , or a fragment, mutataion or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity; and
- ii) a gene of interest in operative association with said nucleotide sequence.

17. A transgenic monocotyledonous plant comprising a gene construct, said gene construct comprising:

- i) a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5 - 11 and 22-26 , or a fragment, mutation or derivative thereof, or a nucleotide sequence that hybridizes to a nucleotide sequence selected from the group consisting of SEQ ID NO's: 5-11 and 22-26, under the following conditions: hybridization in 5XSSC and 50% formamide at 42°C; and washing from about 0.5XSSC to about 0.2XSSC at 65°C, and exhibits regulatory element activity; and
- ii) a gene of interest in operative association with said nucleotide sequence.

18. A method of expressing a gene of interest within a plant comprising:

- i) operatively linking a gene of interest for which expression is desired with the regulatory region of claim 1 to produce a gene construct; and
- ii) introducing said gene construct into said plant and allowing for expression of said gene of interest.

19. The method of claim 18 wherein said plant is a monocotyledonous plant.

20. The method of claim 18 wherein said plant is a dicotyledonous plant.

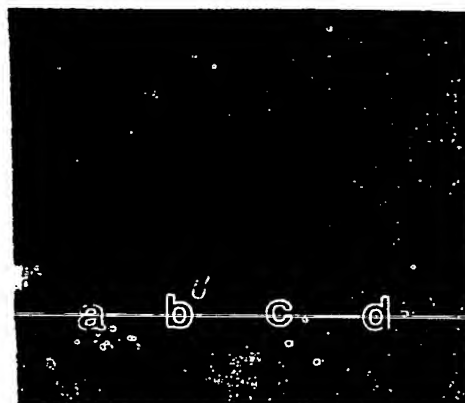


FIGURE 1

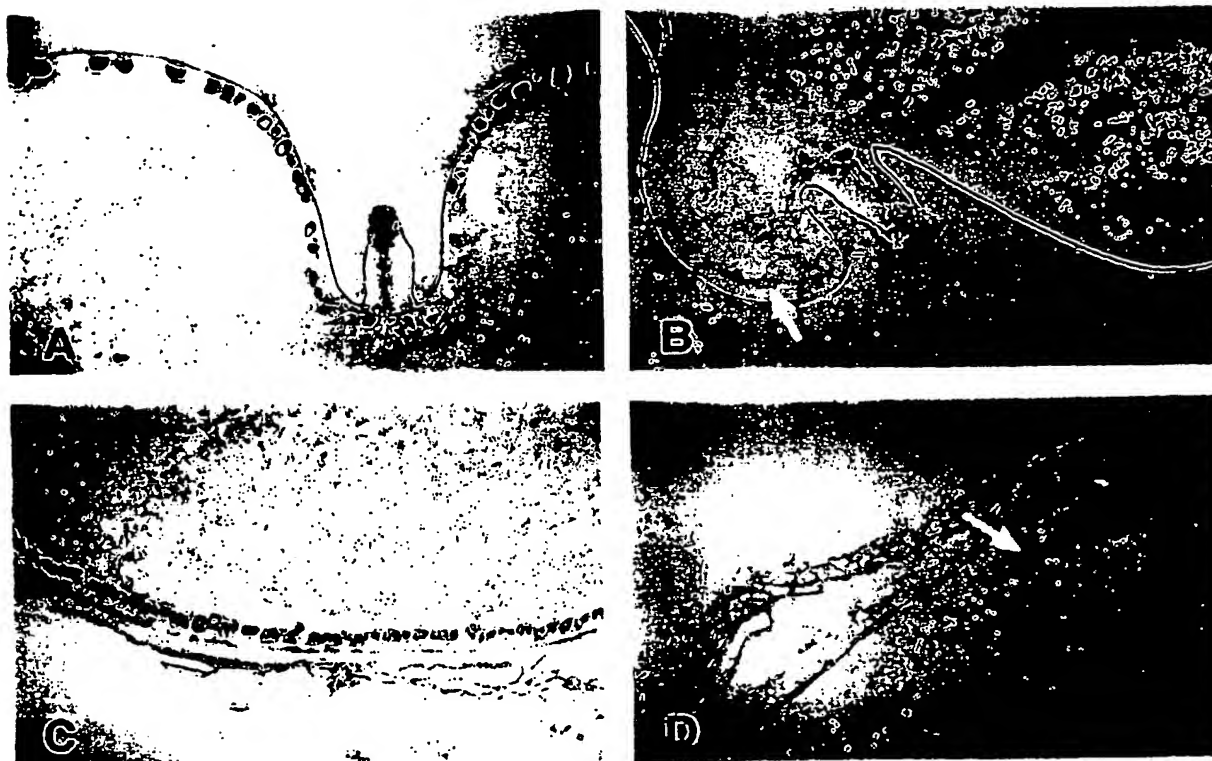


FIGURE 2

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*SEQ ID NO:1 (687 bp) >
-688 TCTAGAGAAAAGAGTTTGTAGACCGGAGGTATTTGTTAGGAAGTACTTCTTGCCATACTAGT
-628 TTCAATAAAGTAGCTTGAAAAGACATTTGTTAAGCAACCATGTGTTTTTAATATGAAGAT
-568 CCTCAATACCGAGAGCCTTTGGTCCCATGGATGACACAAAACCTTCCCACTTGTTTTTTTT
*SEQ ID NO:2 (473 bp) >
-508 TTTTGTGTGTGTGTGGGTAAACTTCCCACTTGGTTAACCTATACTTCCGCTTATGTTTCAT
-448 CACTTTGCCAGAAAAATTGCATATGTGAAGGAAGTGCCAATATTTAATACCGTCTGGTGTT
-388 ATAAATTCATCTCCCAAAATTATTGGAGTTGAAGATTCACCTTGAAAAATAATTTGACAT
-328 ATTAAGATGTTGCCCTTGCGCGGGGTATCTGCAAATTGAGGATCCAAGGGACGATTGCA
-268 TCCAGTTCTAAACACACCATTATGATTTCACTGATAATGCATGCTTCCAAAGCCCAGCTG
*SEQ ID NO:3 (206 bp) >
-208 CAAGCTTGGGCCATCCTTCGGAAGGGAAAAAGAAAAAGGGGTCCTGCTGCACCAGCGACT
-148 AAACCATCCACGCATCTCTCGCTCGAACCCCTATTTAAAGCCCCTCCATTCTTCCCTACAT
-88 TCTCCACACAAACCACGAGTTGCTCATCTCTCCACCCAATCATCACTAGCTAATACGGTGC
*+1
-28 ACTGTTAGCTACAGACCAAGAAGTGATCATGCCCCGCGCTCAGGTAATGCTCATGGCCGT
33 CGCCTTGGTGCTCATGCTCGCGGGCGGTCCCGCGCGCTGCCGTGGCCATCGACTGCGGCCA
93 CGTTGACAGCTTGGTGAGACCCTGCCTGAGCTACGTTCAGGGCGGCCCCGGCCCCGTCTGG
153 GCAGTGCTGCGACGGCGTCAAGAACCTCCATAACCAGGCGCGATCCCAGAGCGATCGCCA
213 AAGCGCTTGCAACTGCCTCAAGGGGATCGCTCGTGGCATCCACAATCTCAACGAGGACAA
273 CGCCCGCAGCATCCCCCCTAAGTGCGGTGTCAACCTCCCATACACCATCAGTCTCAACAT
333 CGACTGCAGCAGGTGATTAATTCACATGCAAGCATATATATGAACACTCATCCACGTA
393 AAATTTATTGATATTAACATTAATCAAATCTTTGCACTGCAGGGTGTAATGGCGGACGAT
453 CCGTCAAGCTGGTGCTCAGCTCATCCATCCACGTGGAGTTGAAGCGCGCAGCCTCTATCC
513 CTATGTAGTATGGTCACTAGTTATGCGAGTTTATACTGAATATGAATAAGAACTCTCTCC
573 AGCTGGCTTGCTGGTACTCCTCTGGAGGAGATCAGTATCTGTGTACCTGAGAGTTGAGAG
633 TTTGTACCATGGGCACTCCCAGTGTATGACTTTAATACATACAACTCGTTCTGTTCAT
693 GCGTGTGACTTATCTTTGTTTCTCAGTTTCGCTGTATATACTCCTTCCATCCGGTAT
753 TAGTTGGCGTTCAAACGGATATATCTAGA

FIGURE 3

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-679 GTTTTAGACCGGAGGTATTTGTTAGGAAGTACTTCTTGCCATACTAGTTTCAATAAAGTA
    |||| | | | | | | | | | | | | | | | | | | | | | |
-700 GTTTGATAACAAAGTAGTAAAAAACTAAAGTATTAAAAAACTGCAGTAATTTTACGTGTA

-619 GCTTGAAAAGACATTGTTAAGCAACCATGTGTTTTTAATATGAAGATCCTCAATACCGA
    | | | | | | | | | | | | | | | | | | | | | | | |
-630 GATAGAAAATACCATGGTTTTTAATATAATAATATTTTTTGCAGTATTCACAAATGTAGAGA

-559 GAGCCTTTGGTCCCATGGATGACACAAAAC.....
    | | | | | | | | | | | | | | | | | | | | | |
-570 AACTGTTTGATTACGCCACATATTACTGCAGTTTAGATCGAGCAAGTACACGGGAAGAAG

-528 .....TCCCACTTGTTTTTTTTTTTTTGTGTGTGTGTGGGTAACTTCCCACTTGGT
    | | | | | | | | | | | | | | | | | | | | | |
-510 ATAACGACGTCCCACCCCTTCTTTTCGCCTTCTCTGTTTTTTAAAAAGAGGTCTGGGGTT

-477 TAACCTATACTTCCGCTTATGTTTCATCACTTTGCCAGAAAATTGCATATGTGAAGGAAGT
    | | | | | | | | | | | | | | | | | | | | | |
-450 AGTTTTTTTCAATACTGCAGTTTTTAAATCACAATTCTTAGAGGCCAACCAACACCTCATT

-417 GCCAATATTTAATACCGTCTGGTGTTATAAATTCATCTCCCAAATTTATTGGAGTTGAAG
    | | | | | | | | | | | | | | | | | | | | | |
-390 GTAAATAAACTATGATAATCTCCAAAACGCAGTATTCTAAAAATACTAC.....

-357 ATTCACCTTGAAAAAATAATTTGACATATTAAAGATGTTGCCCTTGCGCGGGGTATCTGCA
    | | | | | | | | | | | | | | | | | | | | | |
-339 .....AAAAATTCTTTGTTATCAAACAGGGCCTAAGGAGTTAAAAAATTTAGCC

-297 AATTGAGGATCCAAGGGACGATTGCATC.....CAGTTCTAAACACACCATTATGATTTT
    | | | | | | | | | | | | | | | | | | | | | |
-289 GTAAGTGAAGTTCGCGGAGGCACCAGCAGCTAGCAGTCATCAACACT.....TGATGGT
    *SEQ ID NO:3 >

-242 AGTGATAATGCATGCTTCCAAAGCCCAGCTGCAAGCTTGGGCCATCCTTCGGAAGGGAAA
    | | | | | | | | | | | | | | | | | | | | | |
-235 TGGCAAAGGCGAGTCGACGTGTCGCGGGGCTCGGCCTGAGCGGGAGATACAATCTGTTCT

-182 AAGAAAAAGGGTCTGCTGCACCAGCGACTAAACCATCCACGCATCTCTCGCTCGAACC
    | | | | | | | | | | | | | | | | | | | | | |
-175 CCAGTAACCCCGTCGATTTGGCCCGCCGACTAAAGCATCCAGGCATCTCTCGCTCGAACC

-122 CCTATTTAAGCCCCTCCATTCTTCCCTACATTCTCCACACAACCACGAGTTGCTCATCTC
    | | | | | | | | | | | | | | | | | | | | | |
-115 CCTATTTAAGCCCCTCCATTCTTCCCAACATTCTCCACACCTCCACGAGTTGC.....
    *

-62 TCCACCCAATCATCACTAGCTAATACGGTGCAGTGTAGCTACAGACCAAGAAGTGATCA
    | | | | | | | | | | | | | | | | | | | | | |
-53 .....TCATCACTAGCTAGTACGTTGTAAGTGTAGCTACAGATTAAGAAGTGATCA

2 TGGCCCCGCGCTCAGGTAATGCTCATGGCCGTCGCCTTGGTGCTCATGCTCGCGGCGGTCC
    | | | | | | | | | | | | | | | | | | | | | |
2 TGGCCCCGCGCTCAGGTAAGTGTGCTCATGGCCGTCGCCTTGGTGCTGATGCTCAGGCGGCCCC

```

FIGURE 4a

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62 CGCGCGCTGCCGTGGCCATCGACTGCGGCCACGTTGACAGCTTGGTGAGACCCTGCCTGA
|||||
62 CGCGCGCTGCCGTGGCCCTCAACTGCGGCCAGGTTGACAGCAAGATGAAACCTTGCCTGA
122 GCTACGTT CAGGGCGGCCCGGCCCGTCTGGGCAGTGCTGCGACGGCGTCAAGAACCTCC
|||||
122 CCTACGTT CAGGGCGGCCCGGCCCGTCCGGCGAATGCTGCAACGGCGTCAGGGATCTCC
182 ATAACCAGGCGCGATCCCAGAGCGATCGCCAAAGCGCTTGCAACTGCCTCAAGGGGATCG
|||||
182 ATAACCAGGCGCAATCCTCGGGCGACCGCCAAACCGTTTGCAACTGCCTGAAGGGGATCG
242 CTCGTGGCATCCACAATCTCAACGAGGACAACGCCCCGAGCATCCCCCCCCAAGTGCGGTG
|||||
242 CTCGCGGCATCCACAATCTCAACCTCAACAACGCCGCCAGCATCCCTCCAAGTGCAATG
302 TCAACCTCCCATACACCATCAGTCTCAACATCGACTGCAGCAGGTGATTAATTCACATGC
|||||
302 TCAACGTCCCATACACCATCAGCCCCGACATCGACTGCTCCAGGTGATTAAATTTACACT
362 AAGCATA.....TATATATGAAC
|||
363 CATCCAGAGTGAAATCTTTAAAAAGAACTATATTTACGAACGGAGTGAGTATATAGGAAC
380 ACTCATCCACGTAAAATTTATTGATATTAACATTAATCAAATCTTTGCA.CTG CAGGGTG
|
423 ATTCATCCACGTAAAATTTGTTGATATTAACATTAACACGCATGATTGACCTGCAGGATT
440 TAATGGGCGACGATCCGTCAAGCTGGTGCTCAGCTCATCCATCCACGTGGAGTTGAAGCG
||
483 TACTGAGCGACGATCCGTCAAGCTGGTGCTCAGCTCATCGATCCACGTGGAGCTGAAGCG
500 CGCAGCCTCTATCCCTATGTAGTATGGTCACTAGTTATG.CGAGTTTATACTGAATATGA
|||||
543 CGCAGCCTCTGTCCCTATGTAGTATGGCTACCAGTTATGCCGAGTTTATGCTGA.....
559 ATAAGAACTCTCTCCAGCTGGCTTGCTGGTACTCCTCTGGAGGAGATCAGTATCTGTGTA
|||||
597 ATAAGAACTCTCTCCT.....GTACTCCTTTGGAGGAGATCAGTATCTATGTA
619 CCTGAGAGTTGAGAGTTTGTACCATGGGCACTCCCACTGTTTATGGACTT
|
645 CGTGAGAGTTGAGAGTTTGTACCATCGGCACTCCCACTGTTTATGGACTA

FIGURE 4a1

```

-681 AAAGAGTTT TAGACCGGAGGTATTT GTTAGGAAGTACTTCTTGCCATACTAGT..TTCAA
      ||           | | | | | | | | | | | | | | | | | | | | | |
-822 AACCGTGGCCTAAAAATAAGCCGATGAGGATAAATAAAATGTGGTGGTACAGTACTTCAA
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-623 TAAAGTAGCTTGAAAAGACATTTGTTAAGCAACCATGTGTTTTTAATATGAAGATCCTCA
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-762 GAGGTTTACTCATCAAGAGGATGCTTTTCCGATGAGCTCTAGTAGTACATCGGACCTCAC
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-563 ATACCGAGAGCCTTTGGTCCCATGGATGACACAAAACCTTCCCACTTGTTTTTTTTTTTTTG
      ||||| | | | | | | | | | | | | | | | | | | | | | | | | | |
-702 ATACCTCCATTGTGGTGAAATATTTTGTGCTCATTTAGTGATGGGTAAATTTTGTATTG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-503 TGTGTGT..GTGGGTAAACTTCCCACTTGGTTAACCTATACTTCCGCTTATGTTTCATCAC
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-642 TCACTCTAGGTTTTTGACATTTTCAAGTTTTGCCACTCTTAGGTTTTGACAAATAATTTCCAT
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-445 TTTGCC.....AGAAAATTGCATATGTGAAGGAAGTGCCAATATTTAATACCGTCTGGTG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-582 TCCGCGGCAAAAGCAAAACAATTTTATTTTACTTTTACCCTCTTAGCTTTCACAATGTA
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-390 TTATAAATTCATCTCCCAAATTATTGGAGTTGAAGATTCAGTTGAAAAAATAATTTGAC
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-522 TCACAAATGCCACTCTAGAAATTC.TGTTTATGCCACAGAATGTGAAAAAAACACTCAC
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-330 ATATTA.....AAGATGTTGCCCTTGCGCGGGGTATCTGCAAATTGAGGATCCAAGGGA
      |||| | | | | | | | | | | | | | | | | | | | | | | | | | |
-463 TTATTTGAAGCCAAGGTGTTTCATGGCATGGAAATGTGACATAAAGTAACGTTCTGTGTATA
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-276 CGATTGCATCCAGT...TCTAAACACACCATTATGATTTCAAGTATAATGCATGCTTCCA
      || | | | | | | | | | | | | | | | | | | | | | | | | | |
-403 AGAAAAAATTGTACTCCTCGTAACAAGAGACGGAAACATCATGAGACAATCGCGTTTGGA
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      *SEQ ID NO:3 >
-219 AAGCCCAGCTGCAAGCTTGGGCCATCCTTCGGAAGGGAAAAAGAAAAGGGGTCTGCTG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-343 AGGCTTTGCATCACCTTTGGATGATGCGCATGAATGG.....AGTCGTCTGCTTG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-159 CACCAGCGACTAAACCATCCACGCATCTCTCGCTCGAACCCCTATTTAAGCCCCTCCATT
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-293 CTAGCCTTCGCCTACCGCCCACTGAGTCCGGGCGGCAACTACCATCGGCGAACGACCCAG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-99 CTTCCCTACATTCTCCACACAACCACGAGTTGCTCATCTCTCCACCCAATCATCACTAG.
      || ||| | | | | | | | | | | | | | | | | | | | | | | |
-233 CTGACCTCTACCGACCGGACTTGAATGCGCTACCTTCGTGAGCGACGATGGCCGCGTACG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      .....
-173 CTGGCGACGTGCCCCCGCATGCATGGCGGCACATGGCGAGCTCAGACCGTGCGTGGCTGG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-40 .....CTAATACGGTGCAGTGTAGCTA
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
-113 CTACAAATACGTACCCCGTGAGTGCCCTAGCTAGAAACTTACACCTGCAACTGCGAGAGC
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |

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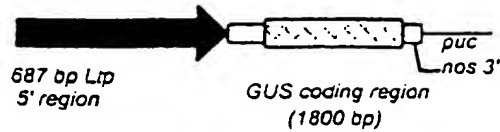
-17 CAGACCAAGAAGTGATCATG
 || || ||
-53 . GAGCGTGTGA.GTGTAGCCGAGTAGATCACCGTACGACGACGACGAGGGGCATG

FIGURE 4b1

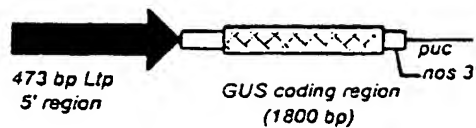
SUBSTITUTE SHEET (RULE 26)

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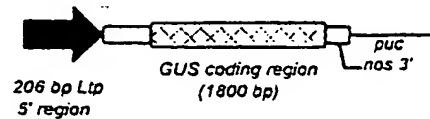
a) p687LtpW1-GUS



b) p473LtpW1-GUS



c) p206LtpW1-GUS



In all three constructs, the ADH1S6 intron lies between
The LtpW1 promoter and the GUS coding region.

d) pLC-GUS

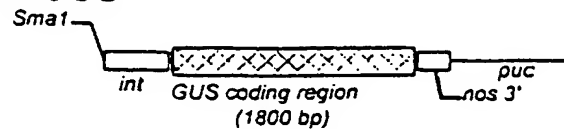


FIGURE 5

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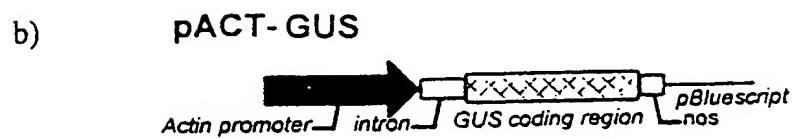
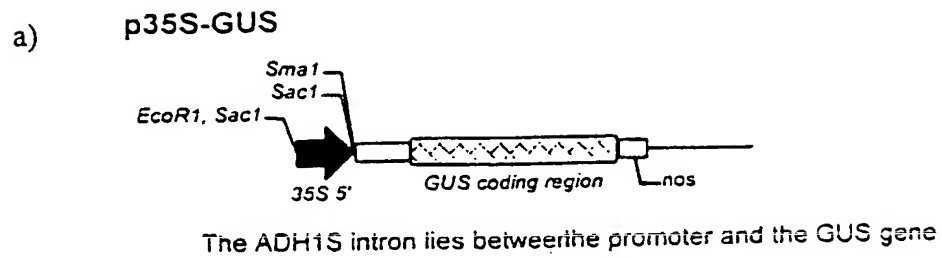


FIGURE 6

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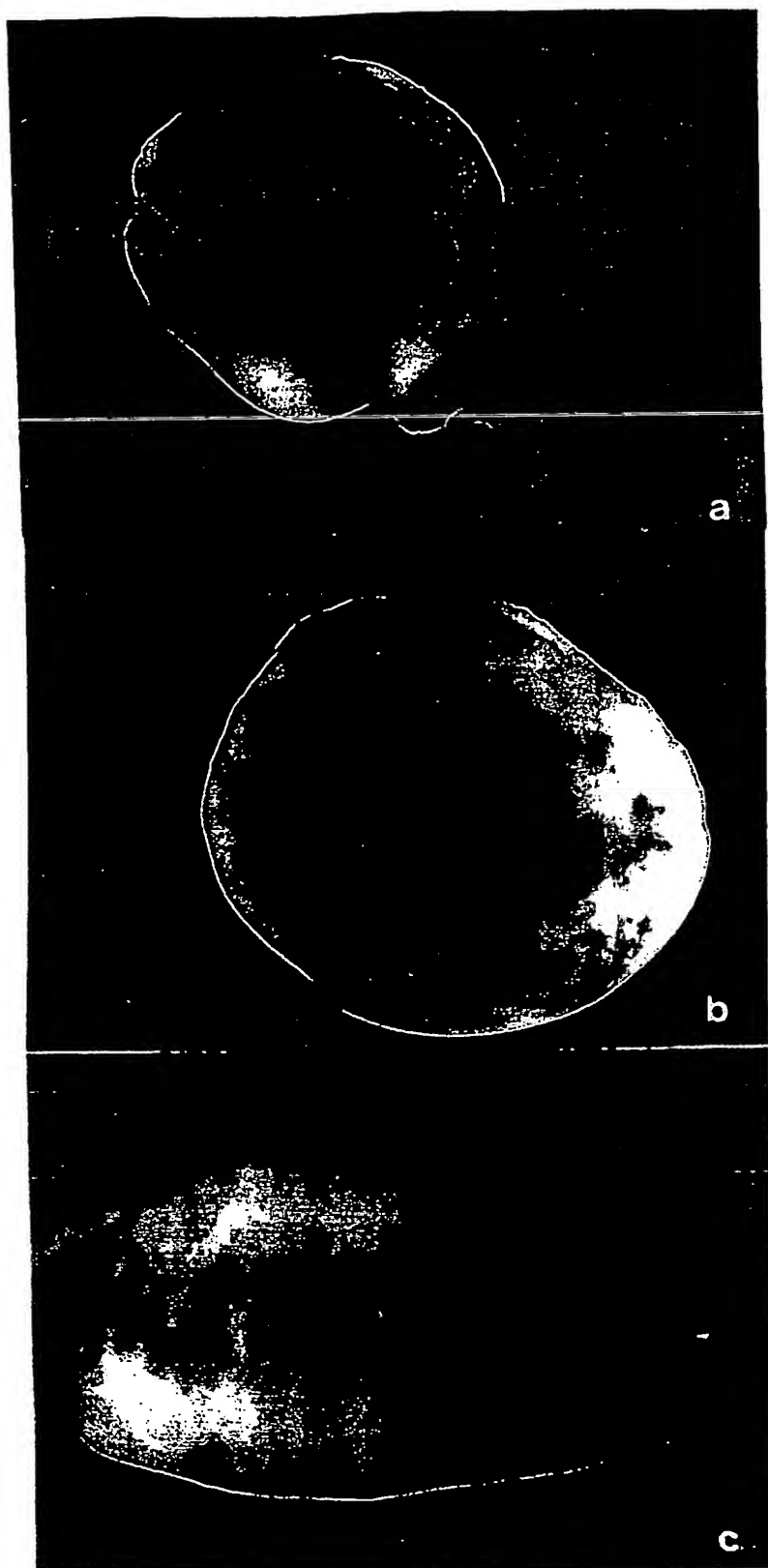


FIGURE 7

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FIGURE 8

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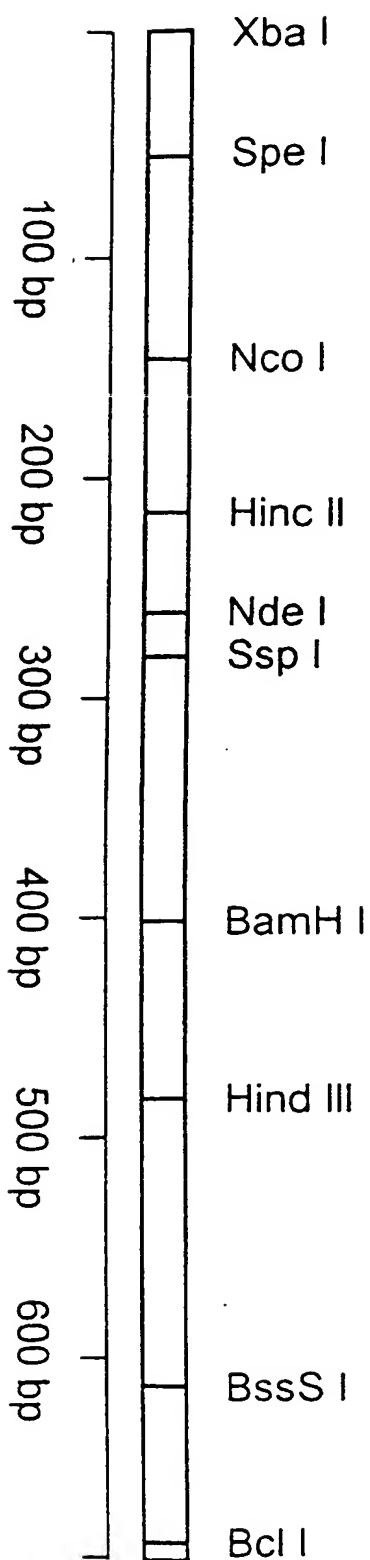


FIGURE 9

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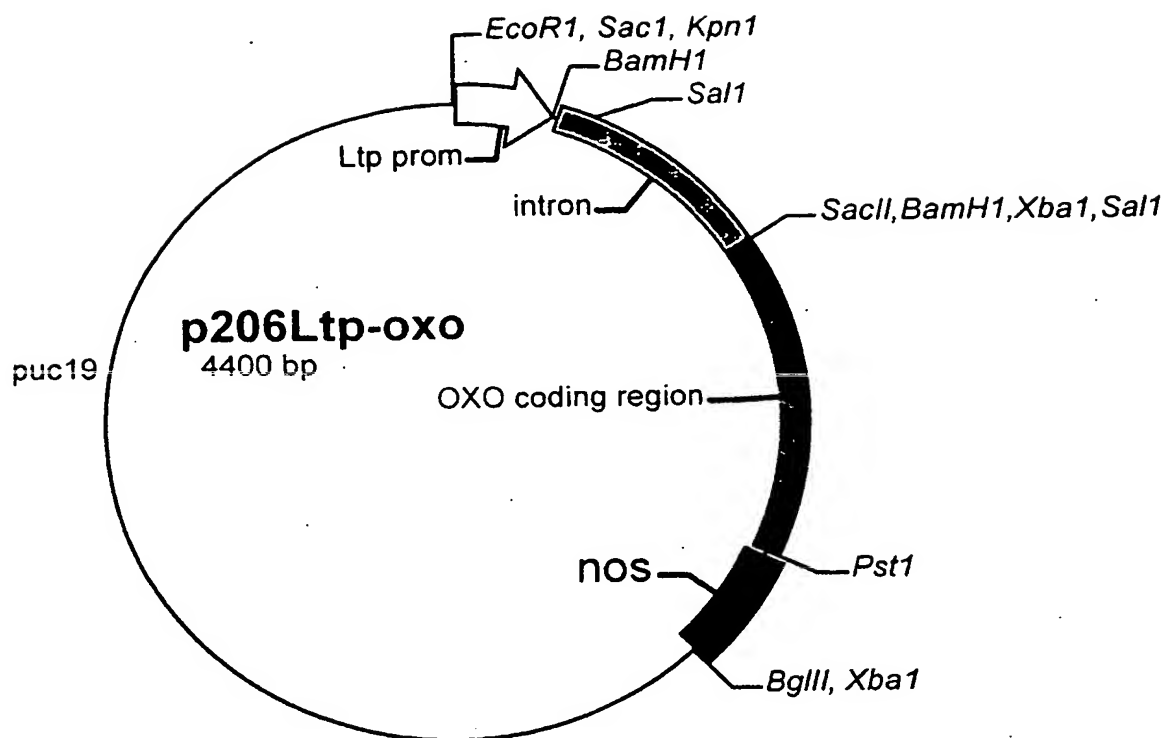


FIGURE 10

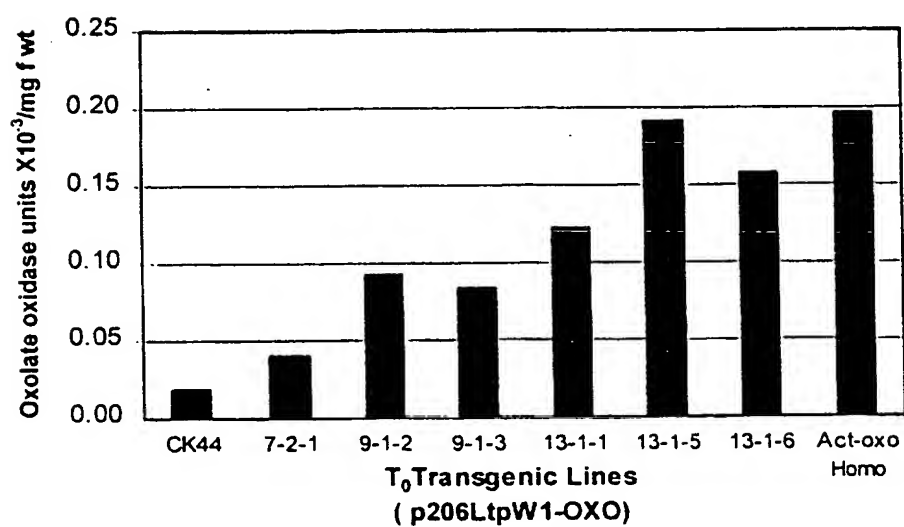


FIGURE 11

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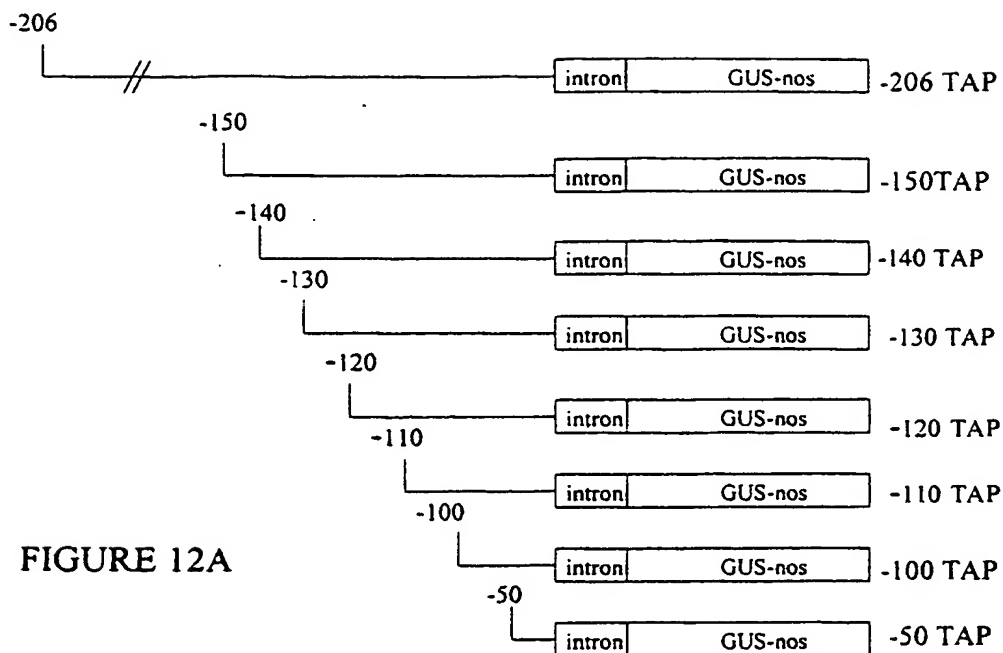
TAP 5' Deletion Series

FIGURE 12A

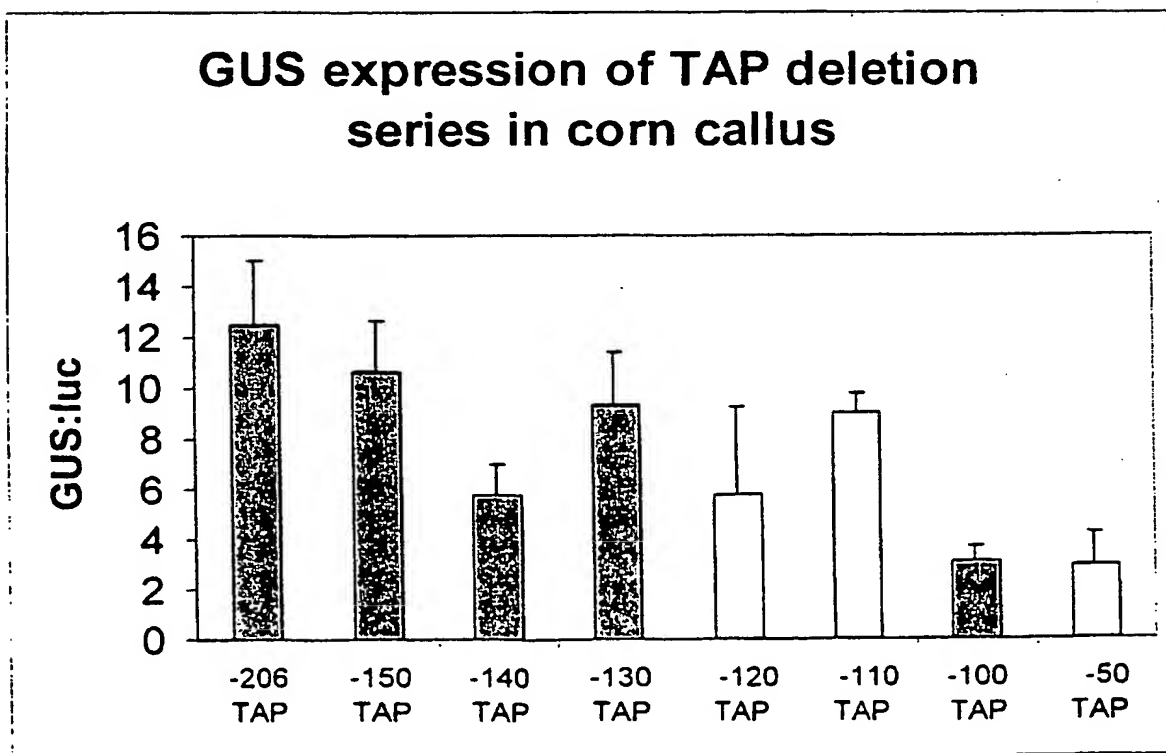


FIGURE 12B

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FIGURE 13A

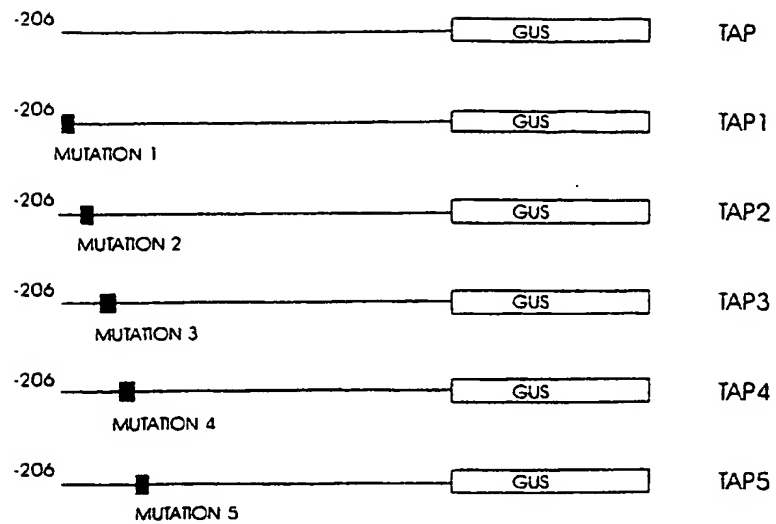


FIGURE 13B

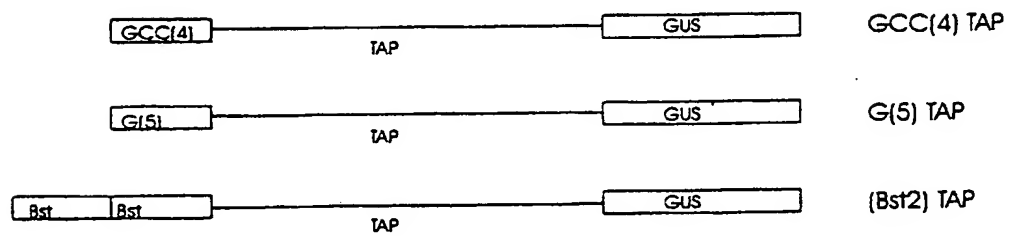
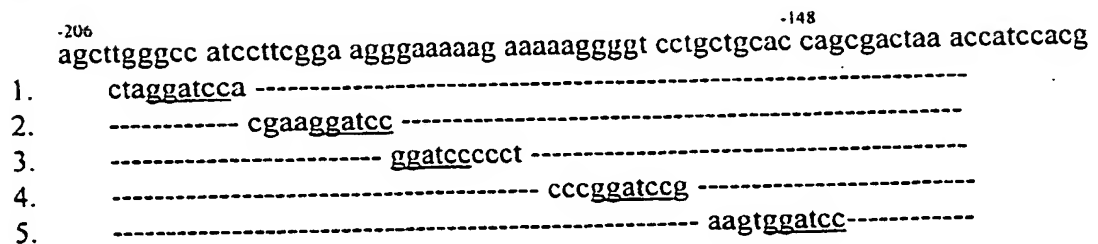


FIGURE 13C



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206 TAP.seq

caagcttggg ccatccttcg gaagggaaaa agaaaaaggg gtcctgctgc accagcgact
 aaaccatcca cgcattcttc gctcgaaccc ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gtcattcttc ccaccaatc atcactagct aatacgggtgc
 actgtagct acagaccaag aagtgatc

150 TAP

aaaccatcca cgcattcttc gctcgaaccc ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gtcattcttc ccaccaatc atcactagct aatacgggtgc
 actgtagct acagaccaag aagtgatc

140 TAP

cgcattcttc gctcgaaccc ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gtcattcttc ccaccaatc atcactagct aatacgggtgc
 actgtagct acagaccaag aagtgatc

130 TAP

gctcgaaccc ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gtcattcttc ccaccaatc atcactagct aatacgggtgc
 actgtagct acagaccaag aagtgatc

120 TAP

ctattttaagc cctccattc ttcctacat
 tctccacaca accacgagtt gtcattcttc ccaccaatc atcactagct aatacgggtgc
 actgtagct acagaccaag aagtgatc

110 TAP

cctccattc ttcctacat
 tctccacaca accacgagtt gtcattcttc ccaccaatc atcactagct aatacgggtgc
 actgtagct acagaccaag aagtgatc

100 TAP

ttcctacat
 tctccacaca accacgagtt gtcattcttc ccaccaatc atcactagct aatacgggtgc
 actgtagct acagaccaag aagtgatc

50 TAP

atcactagct aatacgggtgc actgtagct acagaccaag aagtgatc

150 (-120 to -80 deletion)

aaaccatcca cgcattcttc gctcgaaccc TGCAT accacgagtt gtcattcttc
 ccaccaatc atcactagct aatacgggtgc actgtagct acagaccaag aagtgatc

150 (-130 to -120 / 113 -116 mutation)

aaaccatcca cgcattcttc gctcgaaccc TGCATCA ctatAtaGgc cctccattc
 ttcctacat tctccacaca accacgagtt gtcattcttc ccaccaatc atcactagct
 aatacgggtgc actgtagct acagaccaag aagtgatc

Note: underline represents a PstI/NsiI fusion site, bold is mutations

FIGURE 13D

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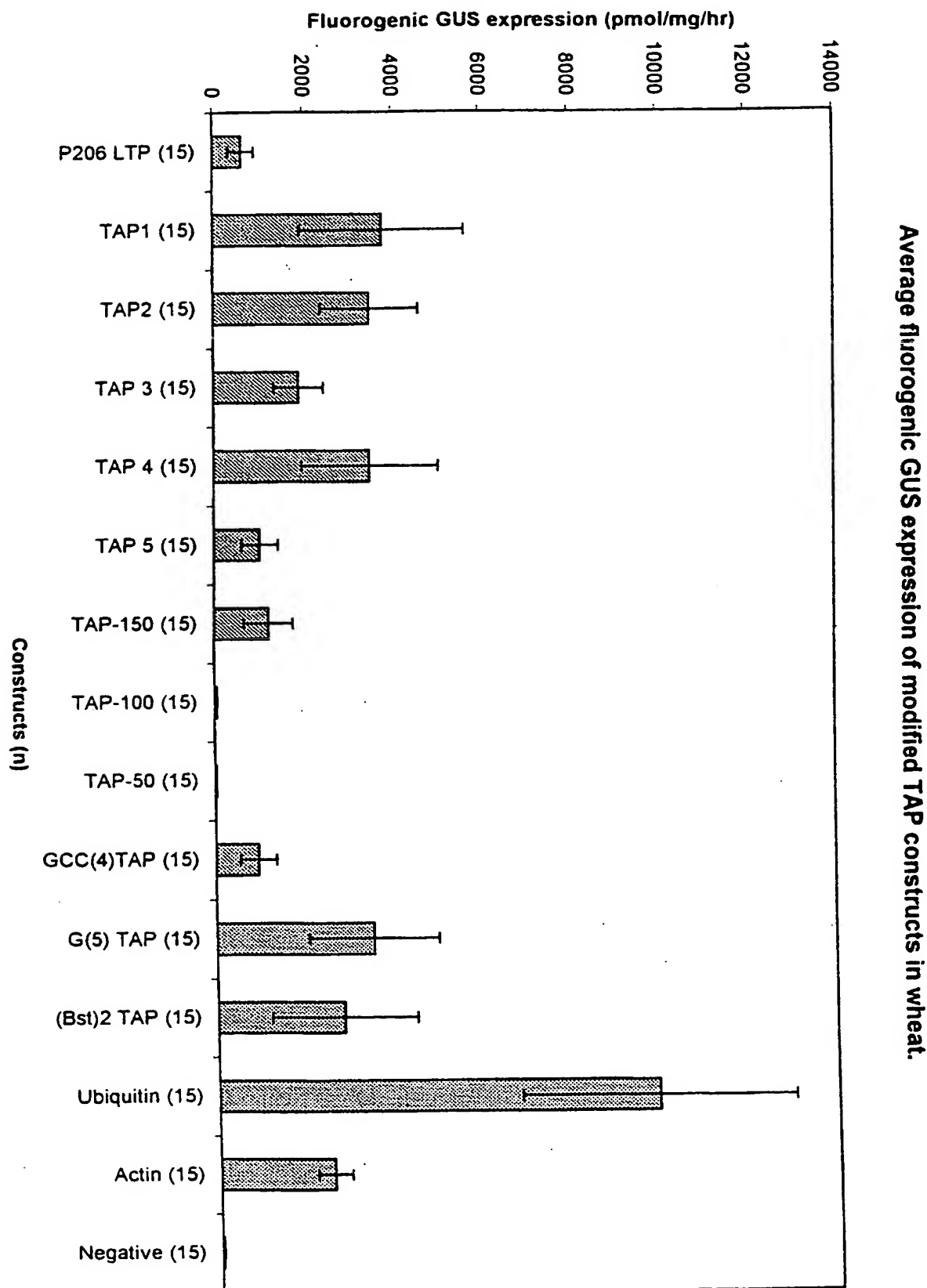


FIGURE 14A

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Mutations/Deletions in -150 TAP

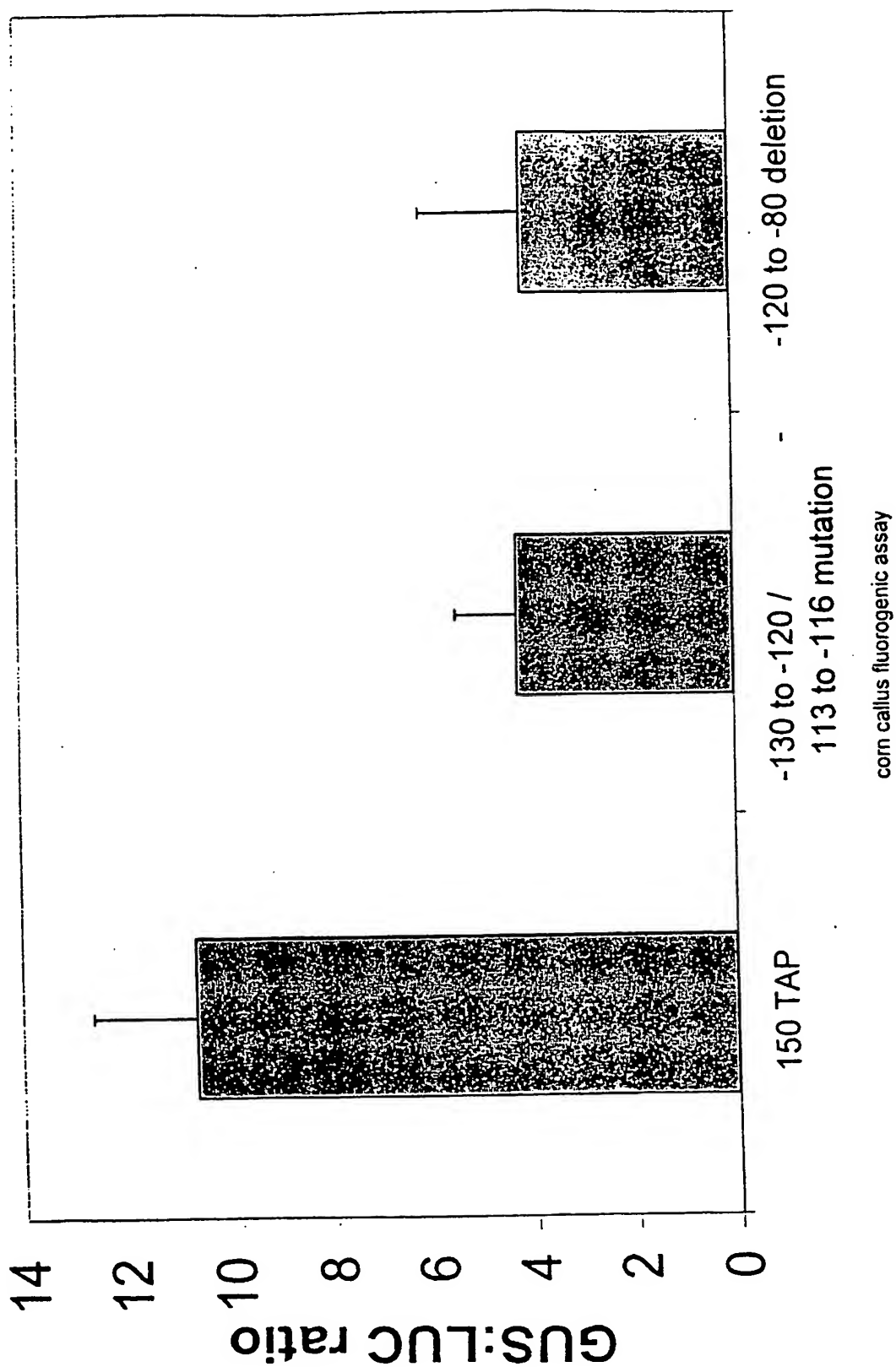


FIGURE 14B

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Gus expression of modified TAP constructs in tobacco

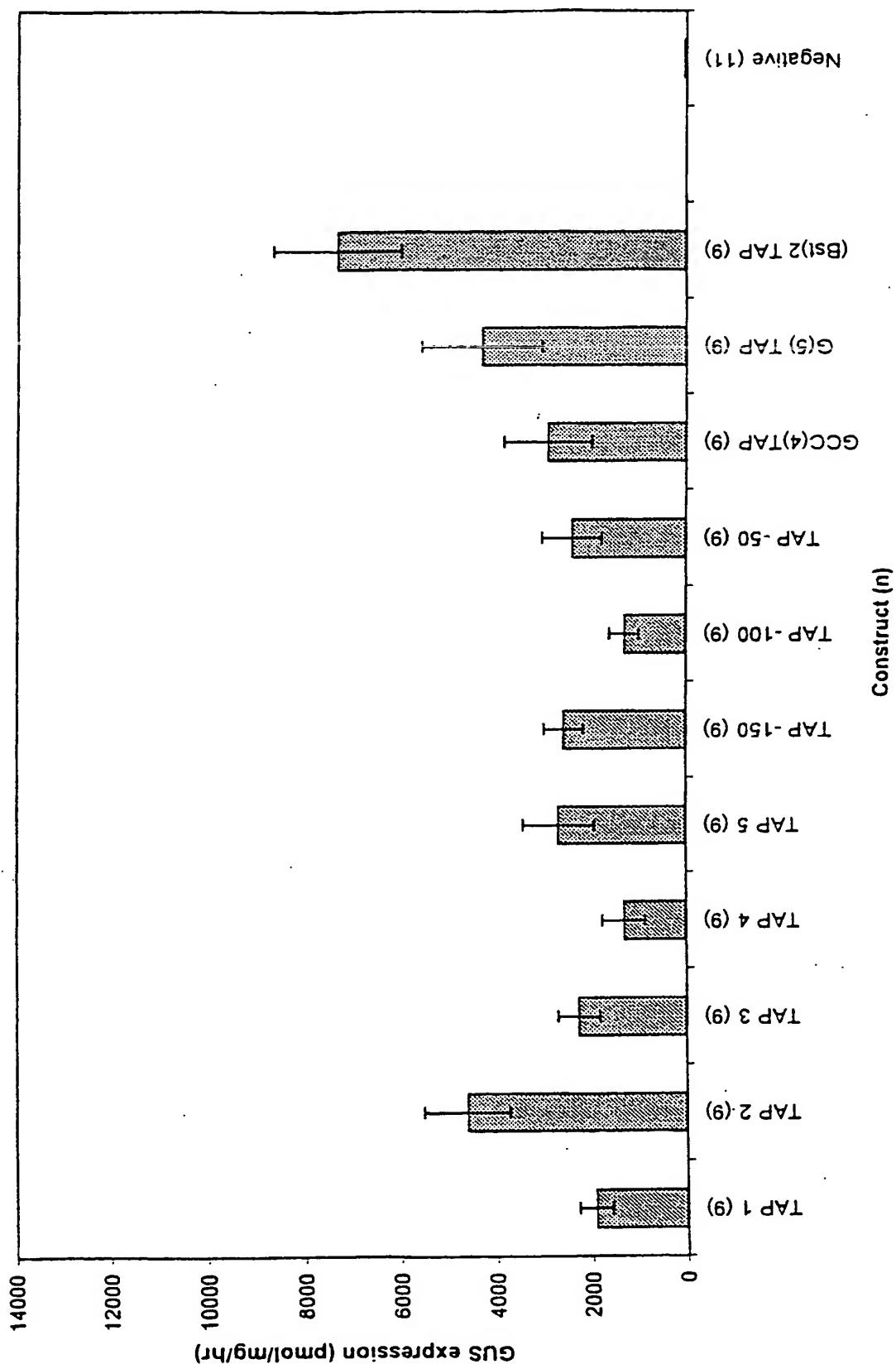


FIGURE 14C

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: John Simmonds
(B) STREET: 130 Amberwood Cres.
(C) CITY: Nepean
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K2E 7H8

(A) NAME: Leslie Cass
(B) STREET: 170 Grey Fox Dr.
(C) CITY: Carp
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K0A 1L0

(A) NAME: Linda Harris
(B) STREET: 6495 Wheatfield Cres.
(C) CITY: Greely
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K4P 1E8

(A) NAME: Sharon Allard
(B) STREET: 39 Wigan Dr.
(C) CITY: Nepean
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K2E 6K9

(ii) TITLE OF INVENTION: Wheat Aleurone Regulatory region

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 687 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: LtpW1

(ix) FEATURE:

(A) NAME/KEY: promoter
(B) LOCATION: 1..687

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTAGAGAAAG AGTTTTAGAC CGGAGGTATT TGTTAGGAAG TACTTCTTGC CATACTAGTT	60
TCAATAAAGT AGCTTGAAAA GACATTTGTT AAGCAACCAT GTGTTTTTAA TATGAAGATC	120
CTCAATACCG AGAGCCTTTG GTCCCATGGA TGACACAAAA CTTCCCACTT GTTTTTTTTT	180
TTTGTGTGTG TGTGGGTAAA CTTCCCACTT GGTAAACCTA TACTTCCGCT TATGTTTCATC	240
ACTTTGCCAG AAAATTGCAT ATGTGAAGGA AGTGCCAATA TTTAATACCG TCTGGTGTTA	300
TAAATTCATC TCCCAAAATT ATTGGAGTTG AAGATTCATC TGAAAAAATA ATTTGACATA	360
TTAAAGATGT TGCCCTTGCG CGGGGTATCT GCAAATTGAG GATCCAAGGG ACGATTGCAT	420
CCAGTTCTAA ACACACCATT ATGATTTTCTG TGATAATGCA TGCTTCCAAA GCCCAGCTGC	480
AAGCTTGGGC CATCCTTCGG AAGGGAAAAA GAAAAAGGGG TCCTGCTGCA CCAGCGACTA	540
AACCATCCAC GCATCTCTCG CTCGAACCCC TATTTAAGCC CCTCCATTCT TCCCTACATT	600
CTCCACACAA CCACGAGTTG CTCATCTCTC CACCCAATCA TCACTAGCTA ATACGGTGCA	660
CTGTTAGCTA CAGACCAAGA AGTGATC	687

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 473 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: LtpW1

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION: 1..473

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AACCTATACT TCCGCTTATG TTCATCACTT TGCCAGAAAA TTGCATATGT GAAGGAAGTG	60
CCAATATTTA ATACCGTCTG GTGTTATAAA TTCATCTCCC AAAATTATTG GAGTTGAAGA	120
TTCACTTGAA AAAATAATTT GACATATTAA AGATGTTGCC CTTGCGCGGG GTATCTGCAA	180
ATTGAGGATC CAAGGGACGA TTGCATCCAG TTCTAAACAC ACCATTATGA TTTCAGTGAT	240
AATGCATGCT TCCAAAGCCC AGCTGCAAGC TTGGGCCATC CTTCGGAAGG GAAAAAGAAA	300
AAGGGGTCCT GCTGCACCAG CGACTAAACC ATCCACGCAT CTCTCGCTCG AACCCCTATT	360
TAAAGCCCTC CATTCTTCCC TACATTCTCC ACACAACCAC GAGTTGCTCA TCTCTCCACC	420
CAATCATCAC TAGCTAATAC GGTGCACTGT TAGCTACAGA CCAAGAAGTG ATC	473

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 206 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
 (B) CLONE: LtpW1

(ix) FEATURE:
 (A) NAME/KEY: promoter
 (B) LOCATION:1..206

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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AGCTTGGGCC ATCCTTCGGA AGGGAAAAAG AAAAAGGGGT CCTGCTGCAC CAGCGACTAA      60
ACCATCCACG CATCTCTCGC TCGAACCCCT ATTTAAGCCC CTCCATTCTT CCCTACATTC      120
TCCACACAAC CACGAGTTGC TCATCTCTCC ACCCAATCAT CACTAGCTAA TACGGTGCAC      180
TGTTAGCTAC AGACCAAGAA GTGATC                                           206
  
```

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1469 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
 (B) CLONE: LtpW1

(ix) FEATURE:
 (A) NAME/KEY: promoter
 (B) LOCATION:1..687

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```

TCTAGAGAAA GAGTTTTAGA CCGGAGGTAT TTGTTAGGAA GTACTTCTTG CCATACTAGT      60
TTCAATAAAG TAGCTTGAAA AGACATTTGT TAAGCAACCA TGTGTTTTTA ATATGAAGAT      120
CCTCAATACC GAGAGCCTTT GGTCCCATGG ATGACACAAA ACTTCCCACT TGTTTTTTTT      180
TTTTGTGTGT GTGTGGGTAA ACTTCCCACT TGGTTAACCT ATACTCCGC TTATGTTTAT      240
CACCTTTTCCCA GAAAATTGCA TATGTGAAGG AAGTGCCAAT ATTTAATACC GTCTGGTGTT      300
ATAAATTCAT CTCCCAAAAT TATTGGAGTT GAAGATTCAC TTGAAAAAAT AATTGACAT      360
ATTAAAGATG TTGCCCTTGC GCGGGGTATC TGCAAATTGA GGATCCAAGG GACGATTGCA      420
TCCAGTTCTA AACACACCAT TATGATTTCA GTGATAATGC ATGCTTCCAA AGCCCAGCTG      480
  
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CAAGCTTGGG	CCATCCTTCG	GAAGGGAAAA	AGAAAAAGGG	GTCTGCTGG	ACCAGCGACT	540
AAACCATCCA	CGCATCTCTC	GCTCGAACCC	CTATTTAAGC	CCCTCCATTC	TTCCCTACAT	600
TCTCCACACA	ACCACGAGTT	GCTCATCTCT	CCACCCAATC	ATCACTAGCT	AATACGGTGC	660
ACTGTTAGCT	ACAGACCAAG	AAGTGATCAT	GGCCCGCGCT	CAGGTAATGC	TCATGGCCGT	720
CGCCTTGGTG	CTCATGCTCG	CGGCGGTCCC	GCGCGCTGCC	GTGGCCATCG	ACTGCGGCCA	780
CGTTGACAGC	TTGGTGAGAC	CCTGCCTGAG	CTACGTTTCA	GGCGGCCCCG	GCCCGTCTGG	840
GCAGTGCTGC	GACGGCGTCA	AGAACCTCCA	TAACCAGGCG	CGATCCCAGA	GCGATCGCCA	900
AAGCGCTTGC	AACTGCCTCA	AGGGGATCGC	TCGTGGCATC	CACAATCTCA	ACGAGGACAA	960
CGCCCGCAGC	ATCCCCCCCC	AGTGCGGTGT	CAACCTCCCA	TACACCATCA	GTCTCAACAT	1020
CGACTGCAGC	AGGTGATTAA	TTCACATGCA	AGCATATATA	TATGAACACT	CATCCACGTA	1080
AAATTTATTG	ATATTAACAT	TAATCAAATC	TTTGCACTGC	AGGGTGTAAT	GGGCGACGAT	1140
CCGTCAAGCT	GGTGCTCAGC	TCATCCATCC	ACGTGGAGTT	GAAGCGCGCA	GCCTCTATCC	1200
CTATGTAGTA	TGGTCACTAG	TTATGCGAGT	TTATACTGAA	TATGAATAAG	AACTCTCTCC	1260
AGCTGGCTTG	CTGGTACTCC	TCTGGAGGAG	ATCAGTATCT	GTGTACCTGA	GAGTTGAGAG	1320
TTTGTACCAT	GGGCACTCCC	AGTGTTTATG	GACTTTAATA	CATACAACTC	GTTCTGTTCA	1380
GCGTGTGACT	TATCTTTGTT	TCCTCACGTT	CGCCTGTCAT	ATACTCCTTC	CATCCGGTAT	1440
TAGTTGGCGT	TCAAACGGAT	ATATCTAGA				1469

INTERNATIONAL SEARCH REPORT

National Application No

T/CA 00/01185

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N15/11 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US 6 013 862 A (ALLARD SHARON ET AL) 11 January 2000 (2000-01-11) the whole document	1-15
P,X	CA 2 230 975 A (CANADA MIN. AGRICULTURE AGRI-FOOD CANADA) 7 November 1999 (1999-11-07) the whole document	1,2,6-8, 10-15
A	WO 95 23230 A (KALLA ROGER ; OLSEN ODD ARNE (NO); LINNESTAD CASPER (NO)) 31 August 1995 (1995-08-31) cited in the application page 4, line 19 -page 9, line 28	1-15
A	WO 95 15389 A (KALLA ROGER ET AL) 8 June 1995 (1995-06-08) the whole document	1-15

-/--



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search

7 March 2001

Date of mailing of the international search report

14/03/2001

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Authorized officer

Schönwasser, D

INTERNATIONAL SEARCH REPORT

Application No

/CA 00/01185

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DIERYCK W ET AL: "NUCLEOTIDE SEQUENCE OF A CDNA ENCODING A LIPID TRANSFER PROTEIN FROM WHEAT (TRITICUM DURUM DESF.)" PLANT MOLECULAR BIOLOGY, vol. 19, 1992, pages 707-709, XP002029148 ISSN: 0167-4412 the whole document</p> <p>-----</p>	1-15

INTERNATIONAL SEARCH REPORT

Patent Application No

PCT/CA 00/01185

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 6013862	A	11-01-2000	NONE		
CA 2230975	A		NONE		
WO 9523230	A	31-08-1995	AU	695526 B	13-08-1998
			AU	1863695 A	11-09-1995
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